THE PREVALENCE AND INTENSITY OF BACTERAEMIA FOLLOWING CONSERVATIVE DENTO-GINGIVAL MANIPULATIVE PROCEDURES IN CHILDREN

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ABSTRACT

The prevalence, intensity and identity of bacteraemia following conservative dento-gingival manipulative procedures in children were investigated. These procedures were placement of a rubber dam, use of the fast and slow drill, placement of a matrix band and wedge and placement of a gingival retraction cord.

Patients and Methods: Two hundred and five children and adolescents undergoing anaesthesia for dental treatment at the Eastman Dental Hospital were recruited. Forty-one subjects were randomly allocated to one of the five procedure groups. Following induction of general anaesthesia, a Y-cannula was inserted into a vein in either the left or right antecubital fossa using aseptic technique. A pre-operative (baseline) blood sample was taken before any dental treatment was carried out. A second blood sample (post-procedure) was taken 30 seconds following the single allocated dento-gingival manipulative procedure. All blood samples were processed using lysis filtration.

Bacterial isolates were identified using both molecular and biochemical techniques. These were 16S ribosomal ribonucleic acid (rRNA) and superoxide dismutase A (*sodA)* partial gene sequencing in addition to carbohydrate fermentation and enzyme hydrolysis and API. Final species identification of streptococci and staphylococci was by *sodA* gene sequencing. The E test was used for antibiotic sensitivity testing.

Results: There was a significantly greater prevalence of bacteraemia compared to baseline following placement of rubber dam ($p = 0.01$), placement of matrix band and wedge ($p = 0.001$) and placement of gingival retraction cord ($p = 0.007$). A significantly greater intensity of bacteraemia compared to baseline was also found following placement of rubber dam ($p = 0.0001$), placement of matrix band and wedge ($p = 0.0001$) and placement of gingival retraction cord ($p = 0.001$). The most frequently isolated bacteria following the dento-gingival manipulative procedures were oral streptococci (51%) and coagulase-negative staphylococci (CNS) (17%). Antibiotic sensitivity testing of oral streptococci revealed high levels of resistance to the macrolides and cephalexin. CNS showed high levels of resistance to penicillin, amoxicillin and the macrolides.

Conclusions: A significantly greater prevalence and intensity of bacteraemia compared to baseline was demonstrated following rubber dam, matrix band and wedge and gingival retraction cord placement. There was a high level of resistance of oral streptococci and CNS to some of the antibiotics recommended by the national bodies for the prophylaxis of infective endocarditis.

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
BHI	Brain heart infusion agar
BLAST	Basic logic alignment search tool
bp	base pair
CBA	Columbia base agar
cfu	colony forming unit
CHD	Congenital heart disease
CNS	Coagulase-negative staphylococci
DNA	Deoxyribonucleic Acid
e-value	Expect value
FAA	Fastidious anaerobe agar
IE	Infective endocarditis
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration for 50% of strains
MIC_{90}	Minimum inhibitory concentration for 90% of strains
NBTV	Nonbacterial thrombotic vegetation
NCCLS	National Committee of Clinical and Laboratory Standards
NCTC	National Culture Type Collection
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
sodA	Superoxide dismutase A
SPS	Sodium polyanetholesulfonate
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA

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CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

INTRODUCTION

The purpose of this work was to investigate the prevalence, intensity and identity of bacteraemia following conservative dento-gingival manipulative procedures. These were placement of rubber dam, the use of fast or slow drill, placement of matrix band and wedge and placement of gingival retraction cord. Earlier guidelines for the prevention of infective endocarditis prepared by the national bodies, the American Heart Association (Dajani et al 1997) and the British Society for Antimicrobial Chemotherapy Endocarditis Working Party (Simmons 1993), were based more on common sense rather than objective data. More recently, a large amount of clinical research has been completed which has demonstrated that, in addition to extractions and scaling, a number of other dento-gingival manipulative procedures cause bacteraemia (Roberts et al 2000). Because of this it was felt that the nature of bacteraemia associated with common restorative procedures should be further investigated. The results from the study reported here have already been used to assist in the formulation of the British Cardiac Society Clinical Practice Committee and Royal College of Physicians Clinical Effectiveness and Evaluation Unit Recommendations for the Prophylaxis of Infective Endocarditis (Roberts et al 2004).

Dental procedures have been implicated in 5-40% of cases of infective endocarditis (Bayliss et al 1983b; Skehan et al 1988; Hollanders et al 1988). The bacteria most commonly associated with both dental procedures and development of infective endocarditis are streptococci and staphylococci (Roberts et al 1997; Karchmer 1999; Roberts et al 2000; Hoen et al 2002; Lucas et al 2002b). The aetiology and pathogenesis of infective endocarditis is complex and involves the interaction of several factors. These include the nature and intensity of bacteraemia, the immune status and the specific cardiac lesion (Masoudi & Sande 1998). The relative importance of each of these factors varies from case to case (Steckelberg & Wilson 1993). In spite of great improvements in diagnosis and treatment, considerable morbidity and mortality continues to result from infective endocarditis (King et al 1988; Sadiq et al 2001).

Although much attention has been given to the prevalence of bacteraemia following various dental procedures, the nature and identity of the organisms are believed to be of greater importance to the development of infective endocarditis (Freedman 1987; Karchmer 1999). The use of quantitative blood culture techniques, such as lysis filtration, not only determine the prevalence and nature of bacteraemia but also the intensity.

General hypotheses

The general hypotheses underlying this study were that bacteraemia after the following procedures was not significantly greater than pre-procedure (baseline) bacteraemia:

1. Placement of a rubber dam
2. Use of a high speed drill
3. Use of a slow speed drill
4. Placement of a matrix band and wedge
5. Placement of a gingival retraction cord

Aims and objectives

The aims and objectives of the study were:

(1) To establish data on the prevalence, intensity and identity of bacteraemia in relation to dento-gingival manipulative procedures associated with conservative dental treatment

(2) To determine the antibiotic resistance of streptococci and staphylococci isolated from the blood following the conservative dental procedures to the antibiotics recommended for prophylaxis by the Endocarditis Working Party (Simmons 1993) and the American Heart Association (Dajani et al 1997)

(3) To use this information to develop evidence-based antibiotic prophylaxis guidelines for patients at risk from developing infective endocarditis

The aims and objectives were achieved by:

(1) Estimation of the percentage prevalence of positive blood cultures from each dento-gingival manipulative procedure at baseline and 30 seconds post-procedure.

(2) Estimation of the number of colony forming units of bacteria per millilitre of blood from each baseline and post-procedure blood sample.

(3) Identification of bacteria isolated from blood cultures by using:

   (a) Biochemical techniques comprising:

      i. Carbohydrate fermentation and enzyme hydrolysis for streptococci
      ii. API ID 32 Staph system for staphylococci

   (b) Molecular techniques comprising:

      i. 16S rRNA partial gene sequencing for all bacteria isolated
      ii. *SodA* partial gene sequencing for streptococci and staphylococci
      iii. Restriction fragment length polymorphism analysis of the *sodA* gene for streptococci

(4) Assessment of antibiotic resistance of oral streptococci, coagulase-negative staphylococci and several other bacterial species to the antibiotics recommended for prophylaxis by the British Society for Antimicrobial Chemotherapy Endocarditis Working Party (Simmons 1993) and the American Heart Association (Dajani et al 1997). These were: penicillin, amoxicillin, erythromycin, azithromycin, clarithromycin, clindamycin, gentamicin, cephalexin, cephalothin, teicoplanin and vancomycin.

REVIEW OF THE LITERATURE

Infective Endocarditis

Infective endocarditis (IE) is an endovascular microbial infection of cardiovascular structures, for example, native valves and ventricular or atrial endocardium. This includes endarteritis of the large intrathoracic vessels, such as patent ductus arteriosus, or of intracardiac foreign bodies for example, prosthetic valves (Horstkotte et al 2004). The heart valves are most often affected; less commonly the lining of the heart chambers and septal defects (Durack & Towns 1998).

The incidence of IE ranges from 10 to 50 cases/million individuals a year (Welsby 1977; Van der Meer et al 1992). The prevalence of IE in the United Kingdom is 2 per 100,000 person-years computed to 1150-1300 cases per year (Skehan et al 1988; Shanson 1989a). In individuals below 30 years of age, the ratio of males to females is 1:1. This increases to 2:1 in those older than 35 years (Van der Meer et al 1992). There is now an increased incidence of degenerative valvular lesions associated with an aging population, hence the increased number of patients with prosthetic heart valves (Dyson et al 1999; Fefer et al 2002). Although morbidity and mortality have decreased due to improvements in antimicrobial and surgical therapy, early death remains high, ranging from 10 to 30% (King et al 1988; Sadiq et al 2001; Tariq et al 2004). In spite of these improvements the incidence of IE has remained unchanged over the last century (Hoesley & Cobbs 1999). IE represents the fourth leading cause of life-threatening infectious disease syndromes after urosepsis, pneumonia and intra-abdominal sepsis (Bayer et al 1998).

Until approximately 40 years ago rheumatic heart disease (RHD) was the most common underlying pathology associated with the development of IE (Noubani & Kulaylat 1989; Bitar et al 2000). Before the introduction of antibiotics, RHD accounted for 70% of cases of IE (Kaye 1994), by the 1970's, 40% (Mostaghim et al 1975), and the current figures range from 5–23% (Bayliss et al 1983b; Hollanders et al 1988; Martin et al 1997). This reflects the declining prevalence of RHD relative to other valvular lesion,s rather than a decreasing incidence of IE in patients with RHD. RHD is still an important predisposing factor for IE in developing countries, for example, in the Lebanon (Kanafani et al 2002) and India (Dhawan et al 1993).

IE occurs less often in children than in adults, accounting for 1 in 1280 paediatric admissions a year (Van Hare et al 1984). In England and Wales the reported prevalence of IE in children is 23-50 cases per million of the population (Young 1987; Skehan et al 1988). In a survey of

30 large cardiovascular centres in North America, over 20% of patients with IE were less than 20 years old (Kaplan et al 1979).

Congenital heart disease (CHD) is now the major underlying lesion in children over the age of 2 years. This is due to the increased survival of children with CHD (Sadiq et al 2001; Ferrieri et al 2002). IE can affect children at any age, but approximately 50% of children are 10 years or older (Johnson & Rhodes 1982). It is unusual below the age of 2 years, although neonatal IE is reported with increasing frequency (Johnson et al 1975). This is related to use of indwelling intravascular catheters in intensive care units and frequently occurs on the right side of the heart (Johnson et al 1975; Oakley 1987). It is associated with disruption of endocardium or valvular endothelial tissues by the catheter induced trauma (Ferrieri et al 2002).

Congenital cardiac abnormalities, particularly cyanotic, have been reported to be the most frequent underlying predisposing lesions in a case series of children with IE (Geva & Frand 1988; Martin et al 1997). The increase in IE in this group is due to their improved survival, more aggressive surgical management and changing approaches to prophylaxis (Gersony & Hayes 1977; Gersony & Hordof 1978). There is an increased susceptibility to infection in lesions associated with high velocity injection of blood. Vegetations are formed on the endothelium which has been damaged by the turbulent blood flow (Behrman & Vaughan 1987). Hence children with a ventricular septal defect and left sided valvular diseases have a higher risk of developing IE than those with, for example, a secundum atrial septal defect (Karchmer 1999).

Classification

Infective endocarditis is currently classified according to the following criteria:

1. The causal microorganism (streptococci, staphylococci, fungi, etc.)
2. Either native valve endocarditis or prosthetic valve endocarditis
3. Acquisition through special circumstances, for example nosocomial IE, intravascular device-related, or intravenous drug abuse

(Kaye 1994)

Aetiology

Normal vascular endothelium is fairly resistant to microbial colonization. Damaged cardiac endothelium is susceptible to infection and the main events leading to IE can be summarized as follows:

1. Damage to the endocardium leads to adherence of platelets and fibrin that results in the development of a nonbacterial thrombotic vegetation (NBTV)
2. A discharge of bacteria into the blood from a local site leads to a transient bacteraemia
3. Adherence of microorganisms to the NBTV with additional deposition of fibrin and platelets (Figure 1)
4. Multiplication of microorganisms within the vegetation
5. Development of the local and systemic consequences of IE

(Freedman 1987)

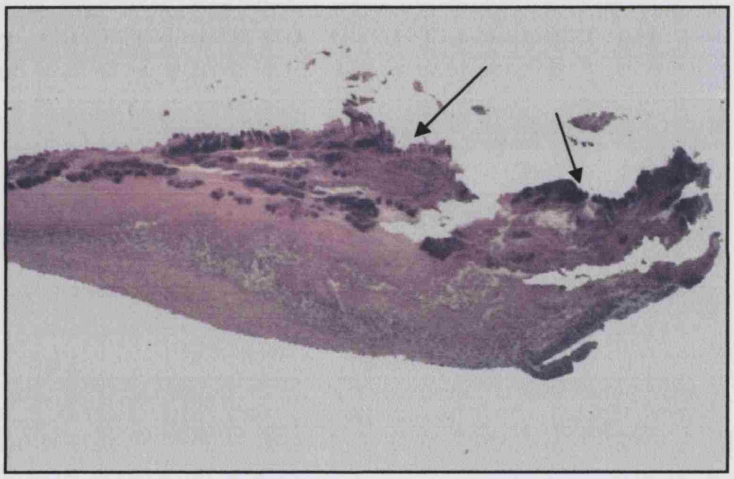


Figure 1: Microscopic appearance of a heart valve with endocarditis

The friable vegetations (arrowed) of platelets and fibrin (pink) are mixed with inflammatory cells and bacterial colonies (blue). The friability explains how portions of the vegetation can break off and embolize
(image from www.nyerrn.com)

Bacteraemia, even in the presence of NBTV, does not invariably cause IE because bacteria must be able to survive in the bloodstream in sufficient numbers to adhere to the endocardium and propagate (Ferrieri et al 2002).

Clinical manifestation

The clinical manifestations of IE are the consequence of

1. Intracardiac tissue destruction due to infection (Figure 2)
2. Embolization of a fragment of the infected vegetation
3. Bacteraemic seeding of remote sites
4. Immune complex injury due to their deposition in tissues and local complement activation

(Karchmer 1999)

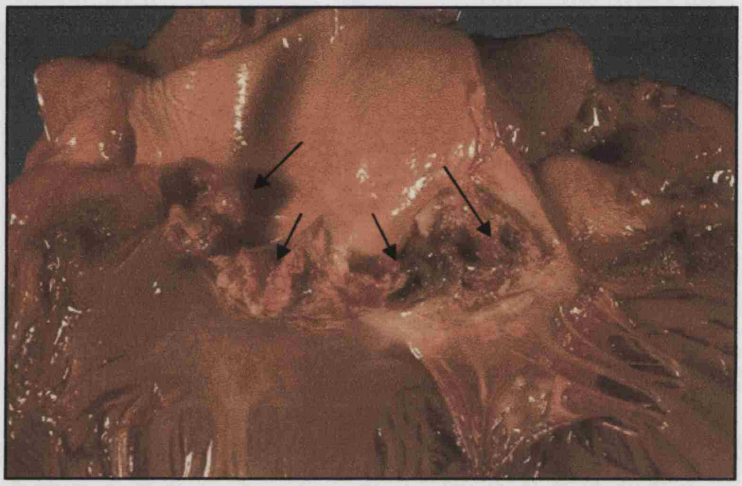


Figure 2: Vegetations on the aortic valve
Irregular reddish tan vegetations (arrowed) overlie valve cusps that are being destroyed. Portions of the vegetation can break off and become septic emboli
(www.nyerrn.com)

Symptoms of IE generally start within 2 weeks of the precipitating event (Kaye 1994; Drangsholt 1998). Fever is present in almost all patients with IE. The onset is gradual with organisms of low pathogenicity, for example the oral streptococci, with mild fever, malaise, weight loss and arthralgia (Masoudi & Sande 1998; Karchmer 1999). With highly pathogenic organisms such as *Staphylococcus aureus*, the onset is acute with high fever, multiple metastatic abscesses, valve destruction and high mortality (Tak et al 2002). Cardiac murmurs are almost always present except in early acute IE or intravenous drug abusers (Kaye 1994).

Petechiae, affecting the conjunctivae, buccal mucosa, palate and upper extremities and splenomegaly each tend to occur in about 30% of cases, mainly of long duration. Osler's nodes occur in 10-25% of patients (Kaye 1994; Hoen et al 2002) and Janeway's lesions in 5-15%. These are most commonly seen in acute staphylococcal endocarditis (Masoudi & Sande 1998; Karchmer 1999). Extracardiac manifestations, such as petechiae and Osler's nodes (Figure 3), are less common in children than in adults (Ferrieri et al 2002).

Figure 3: Common peripheral manifestations of IE

A) Splinter haemorrhages are normally seen under the fingernails or toenails. They are usually linear and red for the first two to three days and brownish thereafter.
B) Conjunctival petechiae
C) Osler's nodes are tender, subcutaneous nodules, often in the pulp of the digits or the thenar eminence.
D) Janeway's lesions are nontender erythematous, haemorrhagic or pustular lesions, often on the palms or soles. (Mylonakis & Calderwood 2001)

Sequelae

These include valvular degeneration, congestive heart failure, pericarditis, renal dysfunction, glomerulonephritis and mycotic aneurysm (Karchmer 1999). Embolic episodes occur in approximately one third of patients (Kaye 1994), which may result in infarction of the brain, myocardium, spleen and kidneys. Heart failure eventually develops and is the most common cause of death (Skehan et al 1988; Hoen et al 2002).

A significant proportion of reported cases of IE have no known predisposing cardiac lesions (Johnson et al 1975; Fernicola & Roberts 1993). Approximately 8 to 15% of paediatric patients with IE do not have a known underlying cardiac lesion or identifiable risk factors. In these cases, infection of the mitral or aortic valve secondary to *Staphylococcus aureus* bacteraemia usually occurs (Johnson et al 1975; Saiman et al 1993).

Dental Procedures and Infective Endocarditis

Dental treatment has been regarded as a major cause of IE. This is because of the high frequency of bacteraemia following various oral procedures and isolation of oral streptococci from cases of IE (Van der Meer et al 1991; Roberts et al 1997). The relationship between dental procedures and IE has been supported by anecdotal clinical reports since 1935 (Okell & Elliott 1935). In addition, many animal models, including rats, rabbits and pigs, have demonstrated that dental bacteria can create histological evidence of IE under experimental conditions (Durack et al 1973; McGowan et al 1983; Overholser et al 1988).

The concept that dental procedures may be intimately associated with the development of IE is based on the following:

1. Symptoms of the disease have been observed in hospital case reports shortly after dental procedures
2. There is a similarity in the majority of cases between the microorganisms responsible for IE and microorganisms found in the oral cavity
3. Many studies indicate that transient bacteraemia occurs frequently following oral procedures

(Lineberger & De Marco 1973)

Epidemiological reports from different countries have shown that 5-40% of IE cases are associated with a possible oral origin (Cherubin & Neu 1971; Bayliss et al 1983a; Skehan et al 1988; Manford et al 1992). In a study of 43 French children diagnosed with IE over a 12 year period, it was reported that 30.5% of the cases were of dental origin (Droz et al 1997).

A survey of IE in the North East Thames Regional Health Authority over a period of 30 months from 1982 to 1984 showed the most frequent single cause for IE was dental treatment within the three months of symptoms in 26 (14%) patients. In 21 (11%) patients, untreated dental disease was present and was thought to be the source of infection. Thus dental treatment or dental disease was implicated in 25% of all cases, with streptococcal infection occurring in 61% of all cases (Skehan et al 1988). In a survey by the Royal College of Physicians Research Unit (Bayliss et al 1986) only 19% of 544 episodes of IE were possibly due to dental procedures (12%) or dental infection (7%). Oral streptococci accounted for 82% of the episodes. A summary of epidemiological information for IE from 1930 to 1996 revealed that 7.5% of patients were exposed to dental procedures and a further 7.5% had dental infections or disease (Drangsholt 1998). As early as 1961, conservative restorative procedures were observed to precede the onset of IE (Harvey & Capone 1961). It is important to note that a chronological relationship between dental or medical procedures and the development of IE does not prove a causal relationship (Van der Meer 2002).

Odontogenic Bacteraemia

Prevalence of Odontogenic Bacteraemia

Transient bacteraemia occurs with manipulation of periodontal tissues and is influenced by the invasiveness and duration of the procedure (Bender & Pressman 1945; Okabe et al 1995). Many workers have demonstrated bacteraemia following dental procedures. These include extraction of teeth (Okell & Elliott 1935; Burket & Burn 1937; Roberts et al 1987; Coulter et al 1990), intraligamental anaesthesia (Roberts et al 1998a), endodontic procedures (Debelian et al 1995), periodontal scaling (Lofthus et al 1991), irrigation of the gingival crevice (Felix et al 1971), periodontal probing (Daly et al 2001) and placement of rubber dam and matrix band and wedge (Roberts et al 2000). Professional cleaning and polishing of teeth and toothbrushing have also been found to cause bacteraemia (De Leo et al 1974; Lucas & Roberts 2000).

A recent investigation provided quantitative data for the prevalence of bacteraemia following a wide variety of dental procedures in children (Roberts et al 1997). A significantly greater prevalence of positive cultures compared with baseline (pre-operative) was demonstrated following toothbrushing, polishing of teeth, scaling, intraligamental injection, rubber dam placement, matrix band and wedge placement, multiple and single extractions and raising a mucoperiosteal flap. The greatest bacteraemia occurred following intraligamental anaesthesia (96.6%) and the lowest after use of the fast drill (4%). Endodontic treatment has been found to be the least likely to cause a detectable bacteraemia (Bender et al 1960; Baumgartner et al 1976). This is because the area of manipulation is limited, with only a

small number of blood vessels open to entry of bacteria (Bender & Montgomery 1986). The percentage of post-procedure blood samples was greater when instrumentation was performed beyond the apex compared with those confined within the root canal (Baumgartner et al 1976; Debelian et al 1995). The incidence of transient bacteraemia following formocresol pulpotomy appears significantly less than that following other oral procedures (4%) (Farrington 1973). Table 1 shows the percentage of positive blood cultures (prevalence) following a variety of dental procedures.

There is evidence that everyday procedures such as chewing or eating are associated with bacteraemia (Cobe 1954; Everett & Hirschmann 1977; Roberts 1997) and have been found to be comparable with certain dental procedures (Seymour et al 2000). It has been suggested that 'everyday' bacteraemia is responsible for so-called random cases of IE (Roberts 1999; Seymour et al 2000).

Table 1: Prevalence of Bacteraemia Following Dental Procedures and Oral Activity

Procedure	Prevalence of Bacteraemia (%)
Extractions	
Single	51
Multiple	68-100
Periodontal Surgery	
Flap procedure	36-88
Gingivectomy	83
Scaling and root planning	8-80
Periodontal prophylaxis	0-40
Conservative Dental Procedures	
Rubber dam placement	29.4
Fast drill	4.3
Slow drill	12.8
Matrix band and wedge placement	32.1
Infiltration analgesia	15.6
Intraligamental analgesia	96.6
Endodontics	
Intracanal instrumentation	0-31
Extracanal instrumentation	0-54
Endodontic Surgery	
Flap rejection	83
Periapical curettage	33
Oral hygiene procedures	
Toothbrushing	0-26
Dental flossing	20-58
Interproximal cleaning - toothpicks	20-40
Irrigation devices	7-50
Chewing	17-51

Adapted from (Seymour et al 2000)

Intensity of Bacteraemia

The intensity of bacteraemia needed to consistently produce IE in the rabbit endocarditis model is 1×10^6 to 1×10^8 colony forming units/ml (cfu/ml) (Bahn et al 1978; Cremieux et al 1993) and this level is species-specific and even strain-specific (Moreillon et al 1988; Masoudi & Sande 1998). Recent work on humans has shown the intensity of bacteraemia to vary from 1 cfu/ml (Coulter et al 1990; Heimdahl et al 1990) to 600 cfu/ml (Roberts et al 1987) following dental extractions (Table 2). The intensity of bacteraemia needed to induce IE is yet unknown.

Table 2: Intensity of Bacteraemia Following Dental Procedures

Procedure	Intensity of Bacteraemia	
	cfu/ml	Range (min-max)
Extractions		
Single	1.12	(0.2 – 6.3) [1]
	0.23	(0 – 4) [2]
Third molar extraction	1.34	(0.34 – 9.88) [1]
Multiple extractions	12.7	(0 – 300) [2]
Mucoperiosteal flap	63.4	(0 – 3200) [2]
Scaling	0.66	(0.25-3.25) [1]
	2.2	(0 – 93) [3]
Polishing teeth	15.9	(0 – 557) [3]
Endodontic treatment	0.54	(0.12 – 1.57) [1]
Conservative dental procedures		
Rubber dam placement	1962	(0 – 100,000) [4]
Fast drill	1.9	(0 – 90) [4]
Slow drill	0.3	(0 – 8) [4]
Matrix band and wedge placement	4.8	(0 – 128) [4]
Intraligamental analgesia	252	(0 – 3018) [6]
Toothbrushing	32.2	(0 – 1666) [3]
Orthodontic treatment		
Separator placement	2.2	(0 – 43.7) [5]
Fitting band	0.3	(0 – 2.8) [5]
Archwire adjustment	0.04	(0 – 0.5) [5]

Data taken from:
1 - (Heimdahl et al 1990), 2 - (Roberts et al 1998b), 3 - (Lucas & Roberts 2000)
4 - (Roberts et al 2000), 5 - (Lucas et al 2002b), 6 - (Roberts et al 1998a)

Bacteraemia in Children

The prevalence of bacteraemia in children is believed to be slightly lower than that in adults. This is likely to be because periodontal disease is rare in children and the number and variety of oral bacteria are less (Matsson 1993; Okabe et al 1995). The incidence of post-extraction bacteraemia in children following dental manipulation has been inconsistent, ranging from zero in some groups of children (Elliott & Dunbar 1968), to 21% (Hess et al 1983b), 38% (Roberts et al 1987), 61% (Peterson & Peacock 1976), 63% (Coulter et al 1990) and 66% (Lucas et al 2001). In more recent investigations, the prevalence in children is comparable to that reported in adults (Heimdahl et al 1990; Okabe et al 1995).

Factors affecting Odontogenic Bacteraemia

The probability and magnitude of bacteraemia associated with dental procedures may depend on the invasive procedure performed (Roberts 1997), the amount of oral inflammation (Lineberger & De Marco 1973) and the number of microorganisms inhabiting the mucosal surface (Rajasuo et al 2004). Investigations of the relationship between the magnitude of dental procedures and the presence of bacteraemia have shown contradictory results. Some workers have reported a significant relationship between the number of teeth extracted and the number of positive post-extraction blood cultures (Robinson et al 1950; Bender et al 1963; Wahlmann et al 1999). Others have found no such relationship (Speck et al 1971; Lineberger & De Marco 1973; Coulter et al 1990; Lockhart 1996).

The issue of whether poor oral hygiene or periodontal health predisposes patients to bacteraemia is controversial. With poor oral hygiene the numbers of bacteria colonizing the teeth increase 2 to 20 fold (Loesche 1997). This increases the number gaining access into the bloodstream leading to an increase in the prevalence and magnitude of bacteraemia (Li et al 2000). Many workers have shown no association between gingival inflammation and bacteraemia (Cobe 1954; Lineberger & De Marco 1973). Others researchers have found that the severity of gingival inflammation has a relationship with the prevalence and magnitude of bacteraemia (Okell & Elliott 1935; Silver et al 1977; Bender et al 1984). Adults with periodontitis have been found to be at greater risk of bacteraemia from periodontal probing (40%) than those with chronic gingivitis (10%) (Daly et al 2001). In a different investigation, no significant difference was found between the prevalence of bacteraemia following extraction of abscessed teeth with inflamed gingivae and the extraction of sound teeth (Peterson & Peacock 1976; Coulter et al 1990). Other workers have found bacteraemia to be more prevalent after extraction of abscessed teeth (Speck et al 1971). In addition, IE has been detected more frequently in periodontally diseased than periodontally healthy rats (Overholser et al 1988).

Duration of Bacteraemia

Animal studies have shown that peak bacteraemia occurs within the first minute after human oral microorganisms are injected into the bloodstream (Silver et al 1975). It has also been shown that the highest yield of microorganisms from blood samples occurs approximately 30 seconds after the onset of dentally induced bacteraemia in humans (Roberts et al 1992; Lucas et al 2001). The reduction of bacteraemia over several minutes is due to the effectiveness of the host defence system (reticuloendothelial system) in rapidly removing microorganisms from the blood (Sell 1996). The transient bacteraemia in most cases is low grade and the blood stream is usually sterile within 20 minutes (Silver et al 1975; Dajani et al 1997; Lucas et al 2001). Occasionally positive blood cultures are seen for up to 1 hour (Jokinen 1970; Rajasuo et al 2004). With modern culturing methods to detect microorganisms in the blood, the duration of bacteraemia may be longer than previously reported (Lockhart 1996).

Entry of Bacteria into the Bloodstream

Research in the 1930's established that microorganisms can enter the blood from the gingival crevice. The investigators painted a broth culture of *Serratia marcesens* onto the gingival margin for a few minutes before extracting the tooth (Burket & Burn 1937). They found that 82.4% of blood cultures were positive for *S. marcesens* following the extraction. Recent studies have validated the source of bacterial dissemination into the vascular component by isolating the same bacteria from pericoronal pockets and in blood after dental extractions (Rajasuo et al 2004) and in the root canal and in blood following endodontic treatment (Debelian et al 1992; Debelian et al 1997).

Oral Flora

The human oral cavity is colonized by a large variety of microorganisms with over 500 different species identified (Moore & Moore 1994; Paster et al 2001) and it is likely that a similar number of uncultivated species may be present (Paster et al 2001; Wilson et al 2002). This diversity is due to the number of different ecological niches in the mouth which are supported by a range of different nutrients (Marsh & Martin 1999). Most organisms in the oral cavity are either facultative or obligate anaerobes, depending on the nature of the microhabitat (Wilson et al 2002). For example, the gingival crevice harbours a high proportion of Gram-negative obligate anaerobes such as *Veillonella* spp. and *Fusobacterium* spp. due to a reduced redox potential and restricted access to oxygen (Listgarten 1994; Kroes et al 1999). The microflora of a freshly colonised tooth surface is dominated by aerobic and facultatively anaerobic bacteria such as streptococci and *Neisseria* spp. (Listgarten 1994). The complex microflora of dental plaque is numerically dominated by *Streptococcus* spp. and *Actinomyces* spp. (Ritz 1967; Babaahmady et al 1997). *Veillionella, Haemophilus, Neisseria, Fusobacterium* and *Propionibacterium* spp. are present in smaller numbers (Babaahmady et al 1997; Paster et al 2001; Wilson et al 2002). Coagulase-negative staphylococci have recently been identified as transient organisms in the oral cavity (Murdoch et al 2004).

Streptococci

Streptococci have been isolated from all sites in the mouth and comprise approximately 20% of the resident oral microflora (Kolenbrander 2000). They are the first organisms to colonize the mouth of neonates. *S. oralis, S. mitis* and *S. salivarius* represent the most commonly isolated of these pioneer species (Smith et al 1993; Pearce et al 1995). Colonization of the oral cavity by other bacterial species gradually occurs as a result of modification of the oral environment by metabolic activities of the pioneer species and the ecological impact of tooth eruption.

The streptococci have undergone considerable taxonomic revision in recent years due largely to the application of molecular and chemotaxonomic approaches (Hardie & Whiley 1994). The general classification of the *Streptococcus* genus was initially divided into pyogenic, viridans, lactic and enteric groups based on physiological characteristics (Sherman 1937). The enterococcal and lactococcal groups, excluding *S. bovis* and *S. equines,* have been transferred to the new genera *Enterococcus* and *Lactococcus*, respectively (Schleifer & Kilpper-Balz 1984; Schleifer & Kilpper-Balz 1987). Six new genera of Gram-positive cocci have been established, the majority of which were split from the *Streptococcus* genus by genetic and phenotypic information. These are: *Abiotrophia* (Kawamura et al 1995a),

Granulicatella (Collins & Lawson 2000), *Dolosicoccus* (Collins et al 1999a), *Facklamia* (Collins et al 1997), *Globicatella* (Collins et al 1992) and *Ignavigranum* (Collins et al 1999b).

Currently the genus *Streptococcus* can be taxonomically divided into six major clusters based on 16S ribosomal RNA (rRNA) gene sequence comparisons (Figure 4) (Kawamura et al 1995b). These are the following:

1. Pyogenic group: *S. agalactiea*, *S. canis*, *S. dysgalactiae*, *S. equi*, *S. iniae*, *S. porcinus* and *S. pyogenes*
2. Bovis group: *S. bovis*, *S. equinus*, *S. gallolyticus*, *S. infantarius*, *S. macedonicus*, *S. waius*, *S. pasteurianus*, *S. suis* and *S. lutetiensis*
3. Mutans group: *S. cricetus*, *S. downei*, *S. mutans* and *S. sobrinus*
4. Anginosus group: *S. anginosus*, *S. constellatus* and *S. intermedius*
5. Mitis group: *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus*, *S. peroris, S. infantis*, *S. orisratti*, *S. australis* and *S. oligofermentans*
6. Salivarius group: *S. salivarius*, *S. vestibularis* and *S. thermophilus*

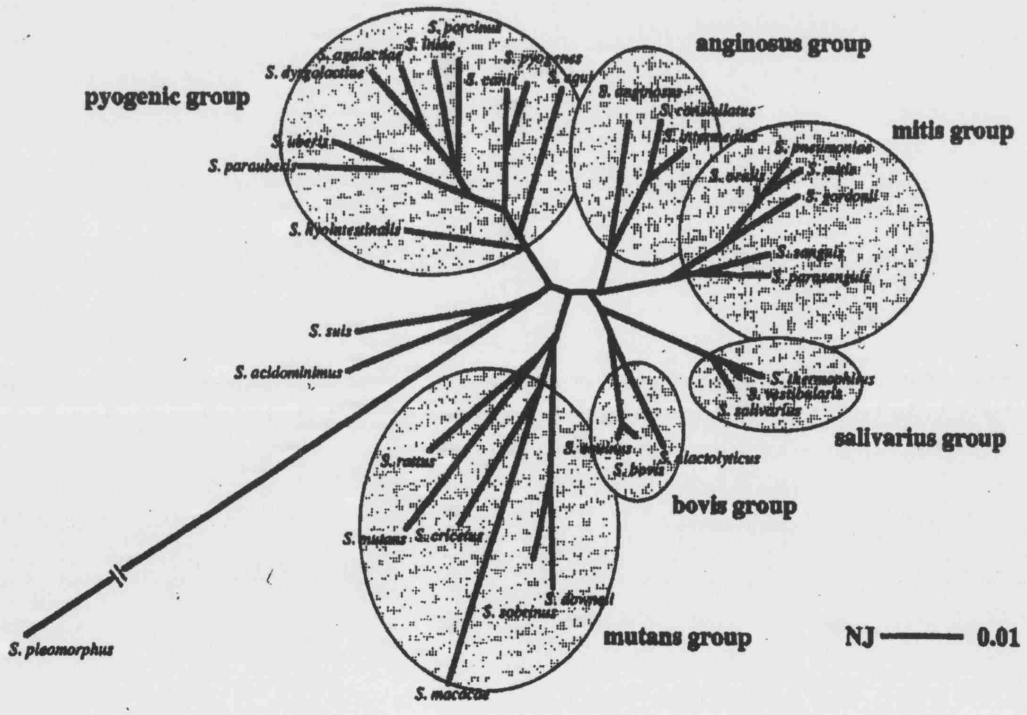


Figure 4: Phylogenetic relationship among 34 *Streptococcus* species
(reproduced from Kawamura et al 1995b)

There are four species groups of oral streptococci. These are mitis, anginosus, mutans and salivarius (Kawamura et al 1995b; Whiley & Beighton 1998). The classification of this group has traditionally been regarded as difficult and many of the new species, amended descriptions and nomenclature changes within the genus *Streptococcus* have occurred in this group (Hardie & Whiley 1994). The term 'viridans streptococci' is sometimes incorrectly used synonymously with oral streptococci as there are many species of viridans streptococci that are not inhabitants of the oral cavity (Whiley & Beighton 1998).

Mutans group

S. mutans was first isolated from carious teeth (Clarke 1924) and from a case of IE (Abercrombie & Scott 1928). Its name derives from the fact that cells can lose their coccal morphology and may appear as short rods or cocco-bacilli (Marsh & Martin 1999). The mutans streptococci are the principle microorganisms implicated in the initiation of caries in humans (Loesche 1986; Becker et al 2002). Detailed biochemical and serological characterisation of *S. mutans* lead to the realization that it was extremely heterogeneous. Many studies in this area lead to the recognition of eight distinct species designated *S. mutans*, *S. sobrinus*, *S. cricetus*, *S. rattus* and *S. ferus* and, more recently, *S. macacae*, *S. downei* and *S. hyovaginalis* (Coykendall 1971; Coykendall 1974; Coykendall et al 1974; Beighton et al 1981; Whiley et al 1988; Devriese et al 1997). The most common species isolated from the human oral cavity are *S. mutans* and *S. sobrinus*, whereas *S. cricetus* and *S. ratti* are rarely isolated (Facklam 2002).

Salivarius Group

Of the species in this group, only strains of *S. salivarius*, *S. vestibularis* and *S. infantarius* have been isolated from humans. *S. salivarius* was first described in 1906 (Andrewes & Horder 1906) and comprises 25% of the pioneer streptococcal species in neonates (Pearce et al 1995). It preferentially colonizes mucosal surfaces in the oral cavity, especially the tongue (Whiley & Beighton 1998). *S. salivarius* has been identified from a variety of human infections (Idigoras et al 2001; Peterson 2002). It has also recently been shown to be associated with caries in children (Becker et al 2002). A similar species to *S. salivarius* was isolated from the vestibular mucosa of the human oral cavity and named *S. vestibularis* (Whiley & Hardie 1988). It has recently been reported to cause prosthetic (Partridge 2000) and native valve endocarditis (Doyuk et al 2002). *S infantarius* is listed under both the bovis and salivarius groups. Some strains are bile-aesculin negative and are therefore excluded from the bovis group (Facklam 2002). *S. infantarius* has recently been isolated from the blood of a patient with IE (Schlegel et al 2000).

Anginosus Group

The three species comprising the anginosus group, *S. anginosus*, *S. intermedius* and *S. constellatus*, were collectively known as '*S. milleri*' (Guthof 1956). The terminology and classification of this group has caused much confusion due to the presence of British (Colman & Williams 1972) and American (Facklam 1977) classification systems at one time. The studies by Colman & Williams (1972) broadened the original description of *S. milleri* by Guthof (1956). Facklam chose to divide the *S. milleri* strains identified by Colman and Williams (1972) into two groups based on lactose fermentation (Facklam 1977). He later regrouped the species based on haemolytic properties with haemolytic strains named *S. anginosus* and non-haemolytic strains divided into *S. intermedius* (lactose fermenters) and *S. constellatus* (lactose non-fermenters) (Facklam 1984). The descriptions of *S. anginosus*, *S. constellatus* and *S. intermedius* have been amended (Beighton et al 1991a) as a result of DNA-DNA hybridization studies (Whiley & Hardie 1989) and phenotypic characterization of clinical and type strains of the *S. milleri* group (Whiley et al 1990a). This group is commonly isolated from dental plaque and mucosal surfaces in humans (Marsh & Martin 1999). *S. anginosus* has been found to be more frequently associated with the development of IE, while *S. constellatus* and *S. intermedius* are more commonly associated with abscess formation (Woo et al 2004).

Mitis Group

The mitis group of streptococci has caused considerable confusion for both clinical microbiologists and taxonomists because of use of invalid species names such as *S. viridans* and *S. mitior* and the use of biotype terminology (Facklam 2002). The biotype describes phenotypic characteristics but does not have official taxonomic status and there are no biotype type strains usually available for study.

S. sanguinis (formerly known as *S. sanguis*) was originally isolated from blood and from heart vegetations of patients with IE (White & Niven 1946). The original description of *S. sanguinis* included production of dextran from sucrose, α-haemolysis on blood agar, hydrolysis of arginine and aesculin and fermentation of sucrose, lactose, salicin and trehalose (White & Niven 1946). *S. oralis* was first designated as a distinct species in 1982 (Bridge & Sneath 1982) and amended in 1985 (Kilpper-Balz et al 1985) and in 1989 following extensive investigation of biochemical and serological activity (Kilian et al 1989). DNA-DNA hybridization confirmed that *S. oralis* was genetically homogeneous and well separated from other species of the group, including *S. mitis*, which had been previously grouped together with *S. oralis* as '*S. mitior*' (Colman & Williams 1972). As a result of this work (Kilian et al 1989) a new species called *S. gordonii* was recognized which included strains formerly

grouped together as '*S. sanguis* subp. *sanguis*' (Coykendall & Specht 1975) and the type strain for *S. mitis*, NCTC 3165. Recent work based on 16S rRNA gene sequence comparison demonstrated 99.93% homology between *S. oralis*, *S. mitis* and *S. pneumoniae*, signifying a very close relationship between these three species and 97% between *S. gordonii* and *S. sanguinis* (Kawamura et al 1995b).

S. cristatus (formerly known as *S. crista*) was described as a *S. sanguinis*-like strain with characteristic tufts of fibrils on the cell wall (Handley et al 1985). A recent investigation found that strains of *S. cristatus* are taxonomically independent at species level, sharing less than 43% DNA-DNA similarity with all established species of the mitis group (Kawamura et al 2000). This confirmed *S. cristatus* as a new taxon within the mitis group.
S. parasanguinis was recognized as an atypical streptococcus isolated from clinical samples (Whiley et al 1990b). Gene sequencing using 16S rRNA showed it to be closely related but distinct from *S. sanguinis* (Kawamura et al 1995b). Recently, *S. peroris* and *S. infantis* (Kawamura et al 1998b), *S. orisratti* (Zhu et al 2000), *S. australis* (Willcox et al 2001) and *S. oligofermentans* (Tong et al 2003) have been added to the mitis group. Whether any of these newly described species are associated with human infections is still unknown.

Staphylococci

Members of the genus *Staphylococcus* are Gram-positive cocci that occur singly, in pairs, tetrads, short chains and irregular grapelike clusters. They are non-motile, non-spore forming and usually catalase-positive (Koneman et al 1997). Most species are facultative anaerobes.

Coagulase-negative staphylococci (CNS) are distinguished from *Staph. aureus* by their inability to clot blood plasma. They were, until 1975, grouped together as *Staph. albus* or *Staph. epidermidis*. The existing classification scheme was extended by adding seven new species to the already known *Staph. epidermidis* and *Staph. saprophyticus* (Kloos & Schleifer 1975). Today there are 32 CNS species with approximately 15 indigenous to humans (Huebner & Goldmann 1999). These include *Staph. epidermidis*, *Staph. capitis*, *Staph. hominis*, *Staph. haemolyticus*, *Staph. warneri*, *Staph. cohnii*, *Staph. simulans*, *Staph. caprae*, *Staph. saccharolyticus*, *Staph. saprophyticus*, *Staph. pasteuri*, *Staph. schleiferi*, *Staph. xylosus*, *Staph. auricularis* and *Staph. lugdunensis* (Kloos & Bannerman 1994; Huebner & Goldmann 1999).

CNS are among the most frequently isolated bacteria in clinical microbiology laboratories (Pfaller & Herwaldt 1988; Kloos & Bannerman 1994). Recently, CNS have been recognized as significant pathogens. *Staph. epidermidis* causes a range of infections, including native

valve endocarditis (Miele et al 2001), prosthetic valve endocarditis (Karchmer et al 1983), osteomyelitis (Paley et al 1986) and mediastinitis (Bor et al 1983). It also causes infections involving permanent pacemakers (Choo et al 1981) and intravascular catheters (Peters et al 1982). *Staph. haemolyticus* is the second most frequently isolated species in clinical laboratories and is implicated in native valve endocarditis (Caputo et al 1987), septicaemia (Gill et al 1983) and peritonitis (Gruer et al 1984). *Staph. warneri*, *Staph. hominis*, *Staph. lugdunensis* and *Staph. schleiferi* have also been implicated in IE (Dan et al 1984; Kralovic et al 1995; Hoen et al 2002).

Staphylococci have been isolated from the oral cavity and are generally regarded as transient colonisers (Dahlen & Wikstrom 1995; Murdoch et al 2004). They have been isolated from plaque from tooth fissures (Theilade et al 1982), supragingival plaque (Percival et al 1991) and subgingival plaque (Rams et al 1990; Dahlen & Wikstrom 1995). Recently workers have revealed that between 94% and 100% of healthy adults (Percival et al 1991; Jackson et al 1999) and 84% of healthy children (Miyake et al 1991) have *Staphylococcus* spp. present in the oral cavity. They make up a very small proportion of the total cultivable subgingival flora, in many cases less than 1% (Rams et al 1990; Dahlen & Wikstrom 1995). The majority of staphylococci isolated from the oral cavity are coagulase-negative (Rams et al 1990; Slots et al 1990; Murdoch et al 2004). The species of staphylococci most frequently reported are *Staph. epidermidis*, *Staph. haemolyticus*, *Staph. hominis*, *Staph. warneri*, *Staph. capitis*, *Staph. saprophyticus*, *Staph. xylosus* and *Staph. simulans* (Rams et al 1990; Murdoch et al 2004).

Staph. epidermidis was the most frequently recovered staphylococci isolated from 46-63% of subgingival samples in periodontitis patients (Rams et al 1990; Dahlen & Wikstrom 1995; Murdoch et al 2004) and from 43% of patients without periodontitis (Murdoch et al 2004). Recently the oral cavity was established as the source of *Staph. epidermidis* bacteraemia in a 15-year old boy following bone marrow transplantation (Kennedy et al 2000).

Actinomyces

The genus *Actinomyces* consists of a heterogeneous group of Gram-positive, mainly facultative anaerobic or microaerophilic rods with various degrees of branching (Koneman et al 1997). At present 15 different *Actinomyces* spp. have been isolated from humans with 10 species found in the oral cavity (Sarkonen et al 2001; Clarridge & Zhang 2002) comprising a considerable proportion of the normal microflora of the mouth (Bowden et al 1975; Ximénez-Fyvie et al 2000). These are *A. naeslundii*, *A. viscosus*, *A. meyeri*, *A. georgiae*, *A. gerensceriae*, *A. israelii*, *A. odontolyticus*, *A. radicidentis*, *A. graevenitizii* and *A. lingnae*. The most frequently detected species in studies of young children are *A. odontolyticus*, *A. naeslundii* and *A. viscosus* (Sarkonen et al 2000; Tanner et al 2002). They have also been isolated in early plaque (Liljemark et al 1993).

A. odontolyticus was the first colonizer and the most frequent *Actinomyces* spp. found on oral mucosal surfaces in infants up to 2 years of age (Sarkonen et al 2000). It was the predominant species in developing plaque in young adults (Liljemark et al 1993). *A. odontolyticus* has been isolated from plaque in young children in significantly greater proportions than from carious lesions (Marchant et al 2001). A soft tissue abscess involving *A. odontolyticus* has been reported recently in an intravenous drug abuser who reported licking his hypodermic needle before injection (Sofianou et al 2004).

A. naeslundii and *A. viscosus* proportions in the dental plaque from infants' mouths has been detected from 6 months and increased gradually with age (Sarkonen et al 2000). In a study carried out on children with mixed dentition, *A. naeslundii* was recovered from 60% and 39% of subgingival plaque of primary and permanent teeth, respectively (Kamma et al 2000). It was also isolated from 76 to 91% of plaque from approximal tooth surfaces in children (Babaahmady et al 1997). *A. naeslundii* has shown a positive association with periodontally healthy teeth with a low mean attachment loss (Liljemark et al 1993). *A. viscosus* has classically been reported to have a different pattern of intraoral colonization to *A. naeslundii*, favouring tooth surfaces to saliva and the tongue (Ellen 1976). *A. viscosus* has been isolated from supragingival plaque in 91% of subjects and from subgingival plaque in 76% (Liljemark et al 1993). In the same study, *A. naeslundii* was isolated from supragingival and subgingival plaque in 54% and 44% of subjects, respectively. *A. viscosus* has been found to be associated with root surface caries (Bowden et al 1990).

A. georgiae, A. gerencseriae and *A. meyeri* have been isolated from the gingival crevices of periodontal healthy individuals (Cato et al 1984; Johnson et al 1990). *A. georgiae* has been

isolated from the teeth of 60% of dentate children under 36 months of age (Tanner et al 2002). *A. radicidentis* has been isolated from infected root canals (Collins et al 2000) and *A. graevenitiz* from infants' saliva (Sarkonen et al 2000). The probable source of *A. lingnae* isolated from bronchial wash, blood and sputum was identified as the oropharynx in a recent investigation (Clarridge & Zhang 2002).

Bacteria Associated with Infective Endocarditis

The predominant bacteria implicated in the aetiology of IE are streptococci and staphylococci (Pallasch & Slots 1996; Drangsholt 1998; Coward et al 2003). They have been reported to cause four out of five cases of IE (Taubert & Dajani 2001). The ability of these organisms to cause IE is due to at least three factors:

1. Their main habitat on the skin and mucous membranes which allows for frequent entry into the blood as random or treatment induced bacteraemia
2. A special ability to adhere to damaged cardiac valves, such as the synthesis of dextrans by streptococci, which increase adhesion of bacteria to valvular tissue (Herzberg et al 1990)
3. An ability to survive and multiply in the NBTV of the cardiac valve

(Pallasch & Slots 1996)

Streptococcus species have traditionally been the most frequently implicated oral microorganisms in the development of IE, comprising 51-63% of organisms detected (Young 1987; Van der Meer et al 1991; Felder et al 1992; Li & Somerville 1998). Over the past ten years the presence of *Staphylococcus* species has increased considerably and, in some groups, has been responsible for almost 50% of cases of IE (Watanakunakorn 1994; Siddiq et al 1996; Mylonakis & Calderwood 2001; Cabell et al 2002). The decline in streptococcal IE reflects a rise in the proportion of intravenous drug abusers (Mathew et al 1995), patients with prosthetic valves (Schulz et al 1996), an increase in nosocomial associated staphylococcal endocarditis (Tak et al 2002; Devlin et al 2004) and IE due to fastidious Gram-negative organisms (Barco 1991).

Oral Streptococci

After one year of age, the 'viridans' group streptococci are the most frequently isolated organisms from patients with IE (Knox & Hunter 1991; Ferrieri et al 2002; Coward et al 2003). Before the introduction of antibiotics, streptococci were implicated in more than 80% of all cases (Cates & Christie 1951) declining recently to 50-60% (Holmstrup et al 2003). Oral streptococci are responsible for 50% of IE in children (Martin et al 1997).

Oral streptococci from both blood cultures and plaque and saliva samples of patients with IE were found to be identical using both conventional microbiological and molecular techniques (Fiehn et al 1995). Recent reference identification of *Streptococcus* species indicate that *S. sanguinis*, *S. oralis*, *S. gordonii*, *S. mitis*, *S. mutans* and *S. salivarius* have been significant causes of IE (Douglas et al 1993). In some studies *S. sanguinis* is reported to account for 50% of all oral streptococci isolated from patients with IE (Roberts et al 1979; Tuazon et al

1986). In a recent study in France, *S. mitis* was found to be the most common oral streptococci isolated from patients with IE (Table 3) (Hoen et al 2002).

IE may also be caused by a group of streptococci that are dependent on L-cysteine or pyridoxal for growth, called nutritionally variant streptococci, subsequently renamed as *Abiotrophia* species (Okada et al 2000). This group, which are members of the human normal flora of the mouth (Kanamoto et al 1996), may account for 5% or more of cases of IE caused by oral streptococci (Ruoff 1991).

Table 3: *Streptococcus* Species Isolated from Patients with IE

Species	(Douglas et al 1993)		(Hoen et al 2002)	
	(n = 47)	%	(n = 68)	%
S. mitis	2	4.2	22	32.4
S. oralis	14	29.8	13	19.1
S. sanguinis	15	31.9	9	13.2
S. mutans	2	4.2	9	13.2
S. pneumoniae	-	-	4	5.9
S. salivarius	2	4.2	3	4.4
S. gordonii	6	12.7	3	4.4
S. parasanguinis	2	4.2	2	2.9
S. constellatus	-	-	1	1.5
S. anginosus	-	-	1	1.5
S. vestibularis	-	-	1	1.5
S. bovis	3	6.4	-	-
Unidentified	1	2.1	-	-

Staphylococci

Staphylococci are more commonly the causal organisms isolated from patients with IE who are intravenous drug abusers (Marsh & Martin 1999; Tak et al 2002). *Staph. aureus* is reported to be responsible for more than 50% of IE in these patients (Kaye 1994). Infections of prosthetic cardiac valves by CNS occurs in 40-60% of cases whereas native valve endocarditis due to CNS is relatively rare, accounting for less than 5% of all native valve infections. Recently, an increase in native valve IE caused by CNS has emerged presenting with acute infection requiring valve replacement (Etienne & Eykyn 1990; Miele et al 2001). *Staphylococcus* spp. endocarditis following dental extractions has also been reported (Etienne et al 1986; Kralovic et al 1995). Table 4 shows the percentage of each *Staphylococcus* spp. isolated from patients with IE (Hoen et al 2002).

Table 4: *Staphylococcus* Species Isolated from Patients with IE

Species	Number of Isolates	Percentage of Isolates
Staph. aureus	90	78
Staph. epidermidis	14	12.2
Staph. lugdunensis	4	3.5
Staph. cohnii	1	0.9
Staph. haemolyticus	1	0.9
Staph. hominis	1	0.9
Staphylococcus spp.	4	3.5

(Hoen et al 2002)

Other Bacteria

An increase in the prevalence of Gram-negative bacteraemia and IE caused by Gram-negative bacteria has been reported over the past 30 years (Barco 1991). Fastidious Gram-negative organisms of primarily oral origin have been recognized as a cause of IE (Barco 1991; Das et al 1997). Collectively these organisms are referred to as HACEK and include *Haemophilus influenzae* (*Haemophilus parainfluenzae*, *Haemophilus aphrophilus*), *Actinobacillus actinomycetomcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella kingae* (Barco 1991; Das et al 1997). HACEK endocarditis has been reported to have increased to 10% for native and 17% for prosthetic heart valves (Geraci & Wilson 1982). In general, IE caused by HACEK organisms is associated with a favourable prognosis (Meyer & Gerding 1988). *Corynebacterium* spp., *Neisseria* spp., *Brucella* spp. and *Bartonella* spp. occasionally cause endocarditis (Karchmer 1999).

Bacteria Isolated Following Dental Procedures

Streptococci have been the most common organisms associated with bacteraemia following dental procedures (Coulter et al 1990; Daly et al 2001). In one study, oral streptococci comprised 85% of the bacteria isolated from blood samples following dental extractions, 40% following third molar surgery, 55% following dental scaling and 15% following endodontic treatment (Heimdahl et al 1990). *S. sanguinis*, *S. oralis*, *S. mitis*, *S. mutans* and *S. intermedius* have been isolated from blood by researchers investigating bacteraemia following dental extractions (Heimdahl et al 1985; Coulter et al 1990; Heimdahl et al 1990; Okabe et al 1995; Roberts et al 1998b). *Actinomyces* spp. have been isolated from blood samples following dental extractions in both children (Coulter et al 1990) and adults (Heimdahl et al 1990). These include *A. israelii*, *A. meyeri*, *A. naeslundii*, *A. odontolyticus*, *A. viscosus* and *A. georgiae* (Heimdahl et al 1990; Rajasuo et al 2004). Recently investigators have shown that 70-88% of bacteria isolated following dental extractions were anaerobes (Heimdahl et al 1990; Okabe et al 1995; Rajasuo et al 2004). The most frequently isolated anaerobes are: *Eubacterium*, *Peptostreptococcus*, *Propionibacterium*, *Actinomyces*, *Fusobacterium*, *Veillonella*, *Lactobacillus* and *Bifidobacterium* (Heimdahl et al 1985; Okabe et al 1995; Rajasuo et al 2004). Among facultative anaerobes and microaerophils the most frequently isolated bacterial genera are: *Streptococcus*, *Staphylococcus*, *Micrococcus* and *Neisseria* (Heimdahl et al 1990; Roberts et al 1997; Lucas & Roberts 2000). Table 5 shows some of the microorganisms isolated following different dental procedures.

Table 5: Microorganisms Isolated from Blood Following Different Dental Procedures

	References							
	Baseline	**Extraction**	**Surgical Extraction**	**Dental Scaling**	**Endodontics**	**Toothbrushing/ polishing**	**Orthodontic procedures**	**Conservative Procedures**
Facultative anaerobes/ aerobes								
Staphylococcus spp.	4, 5, 6	4, 5, 9, 10	1, 4	5		5	6	7
Streptococcus spp.	4, 5, 6, 7	4, 9, 10, 11	1,2,4,	1, 5	1,3	5, 12	6, 8	7
Actinomyces spp.	6	1, 10, 11	1, 2	1	3		6	
Neisseria spp.		4	2, 4	5	1	5		7
Haemophilus spp.		4, 11		1				
Corynebacterium spp.	6	4, 9, 10, 11	1			5		
Enterococcus faecalis			1					
Micrococcus spp.	6	9, 10	1		1		6	7
Anaerobes								
Peptostreptococcus spp.		10	1, 2	5	3	12		
Propionibacterium spp.		4, 10		1	3		6	
Veillionella spp.		1, 4, 10, 11	1, 2, 4	1, 5		5, 12		7
Lactobacillus spp.		1, 10, 11	1, 2				6	
Eubacterium spp.		1, 10	1, 2					
Fusobacterium spp.		10, 11	2	1	3	12		
Bacteroides spp.		1, 4, 11	1, 4	1	1	12		7
Clostridium spp.		1						
Bifidobacterium spp.		10	1					
Capnocytophaga spp.		11	2					
Prevotella spp.		10	2		3			
Poryphyromonas spp.		10						

1 – (Heimdahl et al 1990), 2 – (Rajasuo et al 2004), 3 – (Debelian et al 1995), 4 – (Roberts et al 1998b), 5 – (Lucas & Roberts 2000); 6 – (Lucas et al 2002b), 7 – (Roberts et al 2000), 8 – (Erverdi et al 1999), 9 – (Heimdahl et al 1985), 10 – (Okabe et al 1995), 11 – (Coulter et al 1990), 12 – (Sconyers et al 1973)

Antibiotic Prophylaxis

The aim of antibiotic prophylaxis is to provide adequate plasma levels for the duration of the bacteraemia and most probably for the initial phases of bacterial adherence and colonization of the NBTV when the microorganisms are still accessible (Titsas & Ferguson 2001; Carmona et al 2002). Guidelines and recommendations for antibiotic prophylaxis have been based on various investigations, including experimental animal models (Bahn et al 1978; Wright & Wilson 1982), pharmacokinetic properties (Fluckiger et al 1994), retrospective clinical studies (Gutschik & Lippert 1990), efficacy of antibiotic prophylaxis (Roberts et al 1987; Hall et al 1996a; Hall et al 1996b), procedure related bacteraemia (Heimdahl et al 1990; Roberts et al 1998b) and bacterial susceptibility tests (Dankert et al 1995).

The most recent guidelines for antibiotic prophylaxis are from the American Heart Association (Dajani et al 1997) and the Task Force on Infective Endocarditis of the European Society of Cardiology (Horstkotte et al 2004). Recommendations for endocarditis prophylaxis in the United Kingdom (UK) were published by the British Society of Antimicrobial Chemotherapy Endocarditis Working Party in 1993 (Simmons 1993) and most recently, the Working Group of the British Cardiac Society Clinical Practice Committee and Royal College of Physicians Clinical Effectiveness and Evaluation Unit (Roberts et al 2004).

CHAPTER 2
GENERAL PATIENTS AND METHODS

All the clinical procedures were carried out by the main investigator (HS). These were subject recruitment, recording of oral health indices, insertion of each cannula, taking blood samples and each dento-gingival manipulative procedure.

Patient Recruitment

Ethical Approval and Consent

Ethical approval was granted by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital (Appendix 1) and the Research and Development Directorate of the University College London Hospitals (Appendix 2).

The procedures were explained to both parent and child. Each parent was given an information sheet (Appendix 3) prior to obtaining written consent (Appendix 4) and verbal assent from the child.

Subjects

Children greater than 17.5 kg weight attending the Victor Goldman Unit at the Eastman Dental Hospital for routine dental treatment comprising extractions, restorations and dento-alveolar surgery were recruited.

Exclusion criteria

Patients were excluded for the following reasons:

1. Chronic medical disorders
2. Predisposing cardiac lesion
3. Antibiotics within the previous 3 months
4. Known viral carriage
5. Haemorrhagic disorders
6. Difficult veins

Clinical Procedures

Oral examination

Dental plaque

Dental plaque was recorded as either present (1) or absent (0) using a modification of the index of O'Leary (Franco et al 1996) by visual examination of each tooth quadrisection (mesiobuccal, distobuccal, mesiolingual and distolingual) to give the plaque score. The plaque index was calculated as the proportion of tooth quadrisections with deposits of dental plaque as a percentage of the total number of primary and permanent tooth quadrisections present.

Gingivitis

The presence or absence of gingival inflammation was recorded using a modification of the index of O'Leary (Franco et al 1996) by visual examination of each tooth quadrisection (mesiobuccal, distobuccal, mesiolingual and distolingual) to give the gingivitis score. The gingivitis index was calculated as the proportion of quadrisections with gingival inflammation as a percentage of the total number of primary and permanent tooth quadrisections present.

Gingival bleeding

Spontaneous gingival bleeding was recorded as either present or absent.

Reproducibility

To assess examiner reliability indices for dental plaque, gingivitis and bleeding were recorded by the main investigator and one other paediatric dentist for 10 children attending the Department of Paediatric Dentistry at the Eastman Dental Hospital.

Collection of Blood Samples

Following attainment of general anaesthesia, a 21 gauge Y-cannula (Wallace, London, UK) was placed in a vein in either the right or left antecubital fossa using aseptic technique. The skin was prepared using 1% povidone iodine solution and 70% isopropyl alcohol BP. A 0.5 ml sample of blood was withdrawn and discarded to void any skin contaminants (Roberts 1997). Using a separate sterile syringe, 6 ml of blood were withdrawn and placed immediately into a sterile universal bottle containing 1.23 ml of 0.35% of sodium polyanetholesulfonate (SPS; Sigma, St. Louis, MO, USA) solution to prevent clotting and to inactivate the natural antibacterial action of the blood (Appendix 5). This was the pre-procedure or baseline sample. The cannula was then flushed with approximately 1 ml of 0.9% w/v sterile physiological saline (Figure 5).

Dento-Gingival Manipulative Procedures

A dento-gingival manipulative procedure was performed prior to any dental treatment. This was to ensure that each procedure to be investigated was completed in isolation thus preventing any subsequent bacteraemia caused by the proposed dental treatment confounding the results. The dento-gingival manipulative procedure was selected by consulting a random number table.

The procedures investigated were as follows:

Group 1: Rubber dam

A clamp was placed on either a fully erupted maxillary or mandibular primary or permanent molar. Six teeth were isolated with a rubber dam, including the clamped tooth (Figure 6).

Group 2: Fast drill

Either a carious primary or permanent molar tooth, or molars planned for extraction were drilled for one minute using a high speed handpiece and a diamond bur with water irrigation.

Group 3: Slow drill

Either a carious primary or permanent molar tooth, or molars planned for extraction were drilled for one minute using a slow speed handpiece with either a round or inverted cone carbide bur (Figure 7).

Group 4: Matrix band and wedge

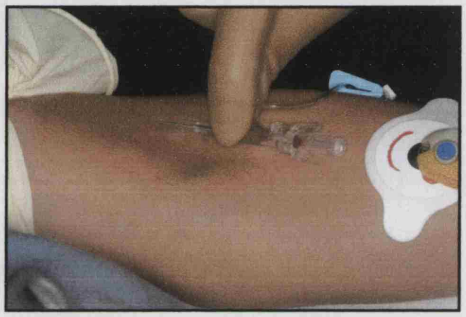
A matrix band was placed on either a mandibular or maxillary primary or permanent molar. A wooden wedge was pushed between the matrix band and the adjacent tooth (Figure 8).

Group 5: Gingival retraction cord

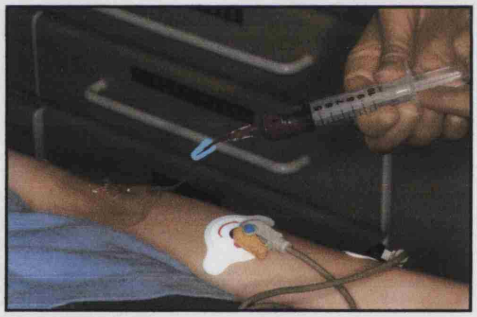
A non-impregnated gingival retraction cord size 1 (Ultrapak™, Ultradent Products, South Jordan, UT, USA) was placed in the gingival sulcus around either a fully erupted maxillary or mandibular permanent molar or a fully erupted maxillary permanent incisor.

Figure 5: Collection of blood samples using aseptic technique

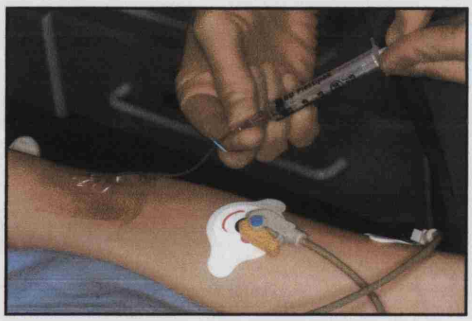
Antecubital fossa cleaned with iodine and isopropyl alcohol



Cannula inserted showing flashback of blood



Blood withdrawal using a sterile syringe



Cannula flushed with saline

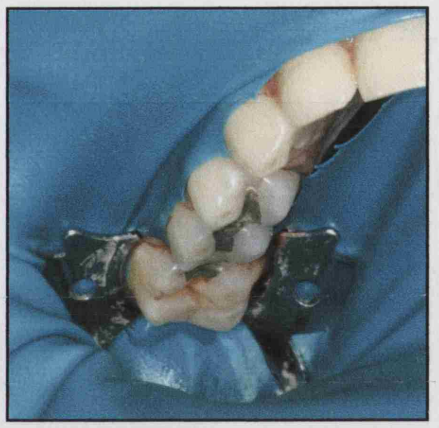


Figure 6: Rubber dam and clamp placed on an upper permanent molar

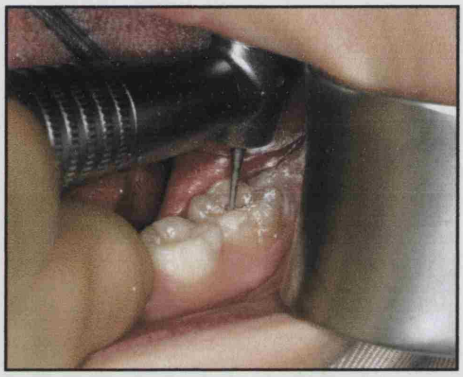


Figure 7: Lower permanent molar drilled using a slow drill

Figure 8: Matrix band and wedge placed around a permanent molar

Thirty seconds after the procedure, a further 6 ml of blood was withdrawn (Roberts et al 1992) and placed into a second sterile universal bottle containing 1.23 ml of 0.35% SPS solution. The cannula was removed and the planned dental treatment was carried out.

Sample Size

The sample size was calculated using results for the prevalence of bacteraemia following placement of rubber dam (Roberts et al 1997). The sample size required to find a difference of 23% with a power of 90% at the 5% significance level was estimated using the equation below.

$$n \geq \frac{F[P_1\%(100-P_1\%) + (P_2\%(100-P_2\%)]}{d^2}$$

n = number required

F = 10.51

d = difference in prevalence 23%

P_1 = Procedure sample 25%

P_2 = Baseline sample 2%

(Altman 1991)

Forty one subjects were needed in each of the five procedure groups to be investigated.

Data Management

All data were entered into an Access 7.0 database. The outcome variables were as follows:

1. The percentage of positive blood cultures (prevalence)
2. The number of colony forming units per ml (cfu/ml) of blood (intensity)
3. The identity of the organisms isolated (speciation)

Statistical Analysis

All data were tested for normality using the Shapiro-Wilk test and found to be not normally distributed. Non-parametric statistical testing was carried out on the clinical data (Altman 1991; Olsen 2003). McNemar's test was used detect any difference in the proportion of positive blood cultures between baseline and post-procedure for each of the five procedure groups. The Wilcoxon signed ranks test was used to compare baseline and post-procedure intensities within the same group. Spearman's rank correlation was used to investigate the association between plaque and gingivitis scores and the prevalence and intensity of bacteraemia. The statistics package used was SPSS for Windows version 11.0 (SPSS Incorporated, Chicago, Illinois, USA).

CHAPTER 3

PREVALENCE AND INTENSITY OF BACTERAEMIA FOLLOWING DENTO-GINGIVAL MANIPULATIVE PROCEDURES

INTRODUCTION

Most clinical laboratories use broth media for blood cultures to enable identification of microorganisms within 24 hours, which is essential for critically ill patients. Broth cultures do not provide the number of microorganisms present in the original blood sample (Yagupsky & Nolte 1990). The technique of lysis filtration not only allows identification of microorganisms, but also the magnitude of bacteraemia following many dental procedures (Heimdahl et al 1990; Lucas et al 2002a; Lucas et al 2002b). This technique has recently been validated (Lucas et al 2002a).

The main techniques for blood cultures are as follows:

1. Pour plate

This technique involves mixing blood with molten agar (at 65°C), allowing it to set and counting the colonies that grow on the agar. It has been used in dental studies and gives reliable quantitative results (Coulter et al 1990) although it is time-consuming and labour intensive (Yagupsky & Nolte 1990).

2. Spread plate

The blood sample is plated directly onto the surface of set agar. This method can only be used for small volumes, usually 1 ml (Santosham & Moxon 1977).

3. Broth Culture

Conventional blood cultures involve inoculation of blood into a liquid medium that supports aerobic and anaerobic bacterial growth. Each bottle is examined daily for macroscopic evidence of microbial growth such as haemolysis, turbidity of media, gas production or formation of discrete colonies (Weinstein 1996). Commercially available automated blood culture systems have been developed which reduce the need for laborious plating of each sample. One of these automated systems is BACTEC (Becton & Dickinson, Maryland, USA). This is based on the utilization of carbohydrate substrates in the culture media and subsequent production of carbon dioxide by microorganisms and its detection by a chemical sensor. The technique can be varied for specific purposes and isolation of specific bacteria by utilizing different media (Shanson 1989b). The disadvantage of this technique is that the intensity of bacteraemia cannot be estimated as the broth encourages rapid growth making it

impossible to know how many bacteria were present in the original sample. It has the advantage of giving a very rapid response (Chokephaibulkit et al 1999).

4. Membrane Filter

This technique was developed to maximize the detection of low magnitude bacteraemia by concentrating the blood into a small area that is easily distributed onto agar plates using a membrane filter (Yagupsky & Nolte 1990). The blood is centrifuged to sediment blood cells and the supernatant aspirated and placed into a plastic filtration unit. The fluid is filtered through a membrane filter using negative pressure and washed with physiological saline by drawing it through the membrane. The filter membrane is removed from the filtration unit and placed onto the surface of agar plates. The disadvantages of this technique are that it is time-consuming and cumbersome and only small volumes of blood can be processed (Yagupsky & Nolte 1990). Differential membrane filtration techniques were developed to allow processing of larger samples of blood and to maximize the recovery of organisms from blood. These techniques are collectively termed lysis concentration and include, lysis centrifugation and lysis filtration.

5. Lysis Concentration: This technique involves lysis of red and white blood cells, concentration of microorganisms by filtration or centrifugation and culture of the concentrate on media (Washington & Ilstrup 1986).

Lysis centrifugation: This technique was developed to recover microorganisms from blood more efficiently (Dorn et al 1979). It was marketed as the Isolator blood culture system (Wampole Laboratories, New Jersey, USA). The Isolator is a special tube containing saponin, a chemical that lyses red and white blood cells. Approximately 7.5 to 10 ml of blood are added to the tube which is mixed by inverting the tube several times and placed into an angle centrifuge and spun at 3000 rpm for 15 minutes to concentrate any microorganisms that may be present. The sediment is aspirated and subcultured onto appropriate media. The Isolator 1.5 Microbial Tube was designed for culturing small volume paediatric blood samples directly onto agar media using the anticoagulant SPS and a saponin containing solution to lyse the cellular components of the blood. Studies comparing this technique with conventional and radiometric broth methods have shown a decrease in time required to obtain isolated colonies with the Isolator system but no significant increase in the recovery of the organisms (Carey 1984; Welch et al 1985). Research on children following oral surgical procedures found the Isolator system to be a poor method for detecting bacteraemia, with only one quarter the sensitivity of the broth culture technique (Roberts et al 1998b; Lucas & Roberts 2000).

Lysis filtration: The basic principle of lysis filtration is disruption of red and white blood cell envelopes by proteolytic enzymes releasing bacteria from within neutrophils. The release of viable organisms from neutrophils increases the yield from blood to a more realistic level (Yagupsky & Nolte 1990). The blood sample is mixed with the anticoagulant SPS because certain bacteria do not survive well in a clot, where phagocytosis can occur. Also, it prevents clogging of the pore filters during filtration (Washington & Ilstrup 1986). SPS inhibits lysozyme, inactivates clinically achievable concentrations of some aminoglycoside and polymyxin antibiotics, inhibits part of the complement cascade and inhibits phagocytosis (Traub & Kleber 1977). The blood sample is then diluted in lysing solution containing 0.08% Na_2CO_3, 0.005% Triton X-100 and 3 ml of commercial streptokinase-streptodornase. This dilution further aids in neutralizing the bactericidal properties of the blood (Washington & Ilstrup 1986) by diluting antimicrobial agents and natural inhibitory factors in the blood to subinhibitory concentrations (Auckenthaler et al 1982). The lysed blood is drawn through a 0.45 μm filter by negative pressure, trapping bacteria on the filter. The filters are placed on agar and incubated aerobically and anaerobically. Lysis filtration has been shown to be more sensitive than conventional blood cultures in rabbit septicaemia (Zierdt et al 1982) and hospital patients (Kagan et al 1977). This technique has been found to be highly sensitive at detecting low level bacteraemia (Hockett et al 1977; Hall et al 1996a), showing a higher frequency and magnitude of bacteraemia than broth cultures (Hockett et al 1977; Lucas et al 2002a). It has been used in many studies of transient bacteraemia following a variety of dental procedures (Heimdahl et al 1985; Heimdahl et al 1990; Debelian et al 1995; Hall et al 1996a; Lucas et al 2002b).

MATERIALS AND METHODS

Lysis Filtration

All blood samples were processed using lysis filtration, within 30 minutes to one hour of collection, in a class 1 microbiological safety cabinet (Figure 9) (BioMat-1, Medical Air Technology, Oldham, UK). Each 6 ml blood sample was added to 190 ml of lysing solution at a pH of 10, containing 0.08% Na_2CO_3 and 0.005% Triton X-100 (Appendix 5). Three millilitres of streptokinase/streptodornase (Varidase™; Wyeth Laboratories, Maidenhead, Berkshire, UK) was added to prevent clogging of the filters during filtration. The lysis solution and blood were mixed thoroughly and incubated aerobically at 37°C for 10 minutes. Each volume was divided into two equal parts so that 3 ml of blood and 95 ml of lysing solution was poured into one filtration unit and the remaining 3 ml of blood and lysis solution into the other. Each filtration unit contains a 47 mm diameter, 0.45 µm filter (Figure 10) (Sartorius AG, Goettingen, Germany). The lysed blood was drawn through the filter by negative pressure of approximately 60 mm Hg.

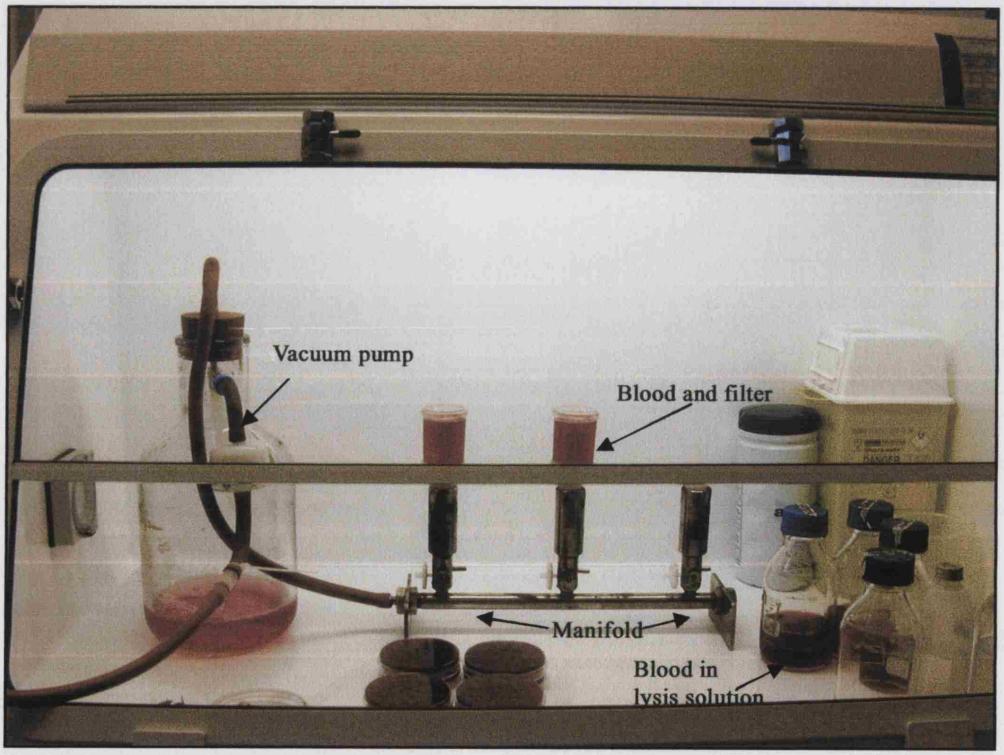


Figure 9: Lysis filtration equipment in a biological safety cabinet

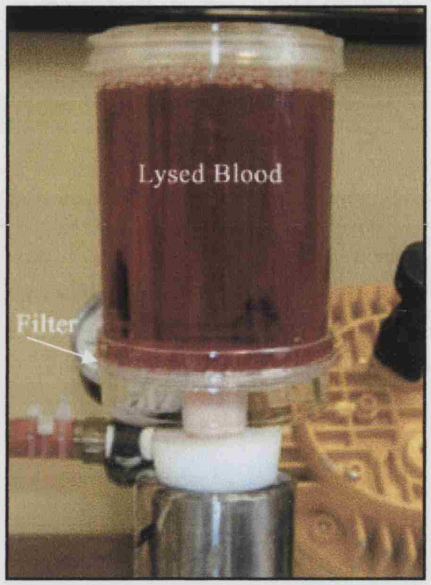


Figure 10: Blood ready to be vacuum filtered

The filter was removed from each subunit using sterile tweezers and placed onto Brain Heart Infusion (BHI) agar (Becton & Dickinson, Oxford, UK) supplemented with 5% defibrinated horse blood. One filter from each blood sample was incubated aerobically and the other anaerobically at 37°C for 10 days. As a negative control, sterile distilled water used to prepare the streptokinase/streptodornase (Varidase™) was filtered through a filtration unit. The filter was then removed from the unit, cut in half using sterile scissors and each half placed on BHI agar. One plate was incubated aerobically and the other anaerobically at 37°C. Non-inoculated plates were included with each filtration run and one plate incubated aerobically and the other anaerobically.

The plates were checked for bacterial growth from the third day for a period of ten days. The number of colonies and their morphology were recorded on each day (Figure 11). Each colony was subcultured onto Columbia agar base (Oxoid, Basingstoke, Hampshire, UK) supplemented with 5% defibrinated horse blood (CBA) for aerobic growth and Fastidious anaerobe agar (Oxoid) supplemented with 5% defibrinated horse blood (FAA) for anaerobic growth. They were incubated aerobically or anaerobically for 24 hours depending on the environment from which the organism had initially been isolated.

The aerobic and anaerobic intensity was calculated by dividing the number of colonies on the aerobic or anaerobic filter by three. The total bacterial intensity was calculated by dividing the number of colonies found on the two filters by six.

Detection Limits

The detection limit for aerobic or anaerobic intensity was 0.33 cfu/ml. The detection limit for total bacterial intensity was 0.17 cfu/ml.

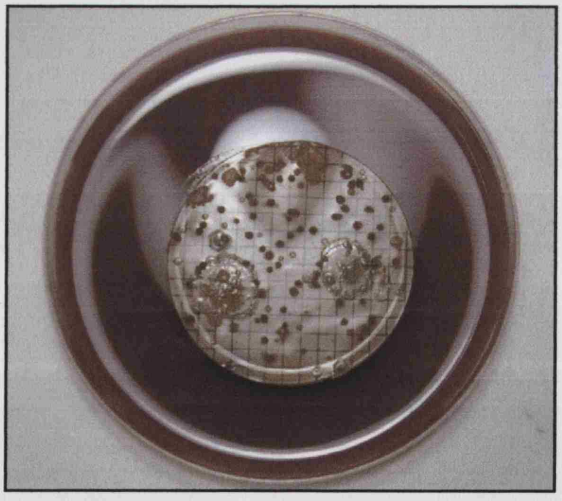


Figure 11: Bacterial growth on filter after 10 days

RESULTS

Clinical Procedures

Reproducibility of Dental Indices

Inter-examiner reliability for recording indices for dental plaque and gingivitis was assessed for 10 children by the main examiner and one other paediatric dentist. The kappa values for plaque showed good agreement and for gingivitis very good agreement between the two examiners (Table 6) (Landis & Koch 1977; Altman 1991).

Table 6: Inter-examiner Reliability

	Kappa
Plaque	**0.733**
Gingivitis	**0.873**

Subjects

From the initial sample of approximately 1400 children and adolescents undergoing general anaesthesia for dental treatment, 205 were recruited for the study. Thirty-six subjects refused to participate and a further six were excluded because of failed venepuncture. Two operating theatres were working at the same time and on occasions two children who had given consent were being treated simultaneously. Because of this five children could not be included. There were 102 males and 103 females and the mean age was 10.8 (sd 3.67), range 4 to 17.5 years.

There were 41 children and adolescents in each of the procedure groups. The teeth involved in the dento-gingival manipulative procedures are shown in Table 7.

Dental Indices

Dental Plaque

The plaque score was recorded for the whole mouth (Table 8) and a separate score was recorded for the teeth involved in each of the gingival manipulative procedures (Table 9). The plaque index was calculated for the whole mouth (Table 8).

Gingivitis

The gingivitis score for the whole mouth (Table 8) and a separate score for the teeth involved in each of the gingival manipulative procedures was recorded (Table 9). The gingivitis index was calculated for the whole mouth (Table 8).

Spontaneous gingival bleeding was not observed in any of the subjects.

Table 7: Teeth Involved in Each Dento-Gingival Manipulative Procedure

Procedure	Upper primary molar	Lower primary molar	Upper permanent molar	Lower permanent molar	Upper permanent central incisor
Rubber dam	7	-	33	1	-
Fast drill	11	18	8	4	-
Slow drill	12	12	8	9	-
Matrix band and wedge	13	2	17	9	-
Gingival retraction cord	-	-	18	2	21

Table 8: Dental Plaque and Gingivitis Scores and Indices: Whole Mouth

(n = 205)	Mean	sd	Median	Min-Max
Plaque score	11.8	8.9	10.0	0 - 60
Plaque index	12.6	9.2	11.4	0 - 54
Gingivitis score	1.2	3.4	0	0 - 32
Gingivitis index	1.2	3.3	0	0 - 30

n = number of subjects

Table 9: Dental Plaque and Gingivitis Scores for Teeth Involved in Each Dento-Gingival Manipulative Procedure (n = 41)

	Plaque Score				Gingivitis Score			
(41 in each group)	Mean	sd	Median	Min-Max	Mean	sd	Median	Min-Max
Rubber dam	1.9	2.2	1.00	0 - 8	0.07	0.3	0	0 - 2
Fast drill	1.2	1.2	1.00	0 - 8	0.10	0.4	0	0 - 2
Slow drill	1.2	0.7	1.00	0 - 3	0.17	0.4	0	0 - 2
Matrix band and wedge	1.3	1.2	1.00	0 - 4	0.05	0.2	0	0 - 1
Gingival retraction cord	0.8	1.1	0	0 - 4	0.29	1.0	0	0 - 6

n = number of subjects

Collection of Blood Samples

General anaesthesia was induced in 173 subjects using intravenous propofol and in a further 32 using nitrous oxide and sevoflurane gas. A laryngeal mask was used for 44 subjects, oral endotracheal intubation for a further 96 subjects and nasotracheal intubation for 65 subjects. Following induction, a Y-cannula was inserted into either the right (n = 75) or left (n = 130) antecubital fossa of each subject using aseptic technique.

Prevalence of Bacteraemia

Aerobic Prevalence

There was a significantly greater prevalence of aerobic bacteraemia compared with baseline following:

1. Placement of rubber dam: baseline 17.1% and post-procedure 41.5% (p = 0.02).
2. Use of slow drill: baseline 4.9% and post-procedure 22% (p = 0.04).
3. Placement of matrix band and wedge: baseline 22% and post-procedure 43.9% (p = 0.02).
4. Placement of gingival retraction cord: baseline 14.6% and post-procedure 41.5% (p = 0.02). Placement of gingival retraction cord was significantly greater than baseline for molar teeth (p = 0.01) but not significant for incisor teeth (p = 0.5).

There was no significant difference in the prevalence of bacteraemia following use of the fast drill.

(Table 10)

Anaerobic Prevalence

There was a significantly greater prevalence of anaerobic bacteraemia compared with baseline following:

1. Placement of rubber dam: baseline 14.6% and post-procedure 39% (p = 0.006).
2. Placement of matrix band and wedge: baseline 14.6% and post-procedure 51.2% (p = 0.0001).
3. Placement of gingival retraction cord: baseline 9.8% and post-procedure 36.6% (p = 0.007). Placement of gingival retraction cord was significantly greater than baseline for molar teeth (p = 0.03) but not significant for incisor teeth (p = 0.06).

There was no significant difference in the prevalence of bacteraemia following the use of the slow or fast drill.

(Table 11)

Table 10: Aerobic Prevalence of Bacteraemia (%) at Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline		Post-procedure		
	%	Number	%	Number	Significance
Rubber dam	17.1	7	41.5	17	**p = 0.02**
Fast drill	9.8	4	17.1	7	p = 0.5
Slow drill	4.9	2	22	9	**p = 0.04**
Matrix band and wedge	22	9	43.9	18	**p = 0.02**
Gingival retraction cord	14.6	6	41.5	17	**p = 0.02**
Molars (n = 20)	20	4	60	12	**p = 0.01**
Incisors (n = 21)	9.5	2	23.8	5	p = 0.5

n = number of subjects

Table 11: Anaerobic Prevalence of Bacteraemia (%) at Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline		Post-procedure		
	%	Number	%	Number	Significance
Rubber dam	14.6	6	39	16	**p = 0.006**
Fast drill	9.8	4	12.2	5	p = 1.0
Slow drill	4.9	2	2.4	1	p = 1.0
Matrix band and wedge	14.6	6	51.2	21	**p = 0.0001**
Gingival retraction cord	9.8	4	36.6	15	**p = 0.007**
Molars (n = 20)	10	2	40	8	**p = 0.03**
Incisors (n = 21)	9.5	2	33.3	7	p = 0.06

n = number of subjects

Total Bacterial Prevalence

There was a significantly greater prevalence of aerobic and anaerobic bacteraemia isolated compared with baseline following:

1. Placement of rubber dam: baseline 29.3% and post-procedure 53.7% ($p = 0.01$).
2. Placement of matrix band and wedge: baseline 31.7% and post-procedure 65.9% ($p = 0.001$).
3. Placement of gingival retraction cord: baseline 22% and post-procedure 56.1% ($p = 0.007$). Placement of gingival retraction cord for molar teeth was significantly greater than baseline ($p = 0.01$) but not significant for incisor teeth ($p = 0.2$).

There was no significant difference in the prevalence of aerobic and anaerobic bacteraemia combined following the use of the slow or fast drill compared with baseline.

(Table 12)

Table 12: Aerobic and Anaerobic Combined Prevalence of Bacteraemia (%): Baseline and Following Each Dento-GingivalManipulative Procedure

Procedure (n = 41)	Baseline		Post-procedure		
	%	Number	%	Number	Significance
Rubber dam	29.3	12	53.7	22	**p = 0.01**
Fast drill	14.6	6	22	9	p = 0.5
Slow drill	9.8	4	22	9	p = 0.2
Matrix band and wedge	31.7	13	65.9	27	**p = 0.001**
Gingival retraction cord	22	9	56.1	23	**p = 0.007**
Molars (n = 20)	23.8	5	66.7	14	**p = 0.01**
Incisors (n = 21)	19	4	42.9	9	p = 0.2

n = number of subjects

Intensity of Bacteraemia

The intensity of bacteraemia was recorded as the sum of the total number of cfu/ml of blood. The median intensity of the whole group including subjects with negative blood cultures (non-detectable bacteraemia) is presented in Tables 13 - 15. The median, minimum and maximum values for subjects with positive blood cultures only (detectable bacteraemia) are presented in the same tables.

Aerobic Intensity of Bacteraemia

There was a significantly greater number of cfu/ml of bacteraemia isolated aerobically compared with baseline following:

1. Placement of rubber dam ($p = 0.005$).
2. Use of slow drill ($p = 0.02$).
3. Placement of matrix band and wedge ($p = 0.002$).
4. Placement of gingival retraction cord ($p = 0.006$). Placement of gingival retraction cord for molar teeth only was significantly greater than baseline ($p = 0.01$) but not significant for the incisor teeth ($p = 0.22$).

There was no significant difference between baseline and post-procedure intensity following the use of the fast drill.

(Table 13)

Anaerobic Intensity of Bacteraemia

There was a significantly greater number of cfu/ml isolated anaerobically compared with baseline following:

1. Placement of rubber dam ($p = 0.001$).
2. Placement of matrix band and wedge ($p = 0.0001$).
3. Placement of gingival retraction cord ($p = 0.005$). Placement of gingival retraction cord for molar teeth ($p = 0.01$) and incisor teeth ($p = 0.03$) was significantly greater than baseline

There was no significant difference between baseline and post-procedure intensity following the use of the slow or fast drill.

(Table 14)

Table 13: Aerobic Intensity of Bacteraemia (cfu/ml): Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min - Max‡	Median	N^{DB}	Median	Min - Max‡	Significance
Rubber dam	nd	7	0.33	0.33 – 0.67	nd	17	0.33	0.33 – 6.33	**p = 0.005**
Fast drill	nd	4	0.5	0.33 – 1.33	nd	7	0.33	0.33 – 0.67	p = 0.6
Slow drill	nd	2	0.33	NC	nd	9	0.33	0.33 – 0.67	**p = 0.02**
Matrix band and wedge	nd	9	0.33	0.33 – 2.0	nd	18	0.5	0.33 – 31.7	**p = 0.002**
Gingival retraction cord	nd	6	0.33	0.33 – 0.67	nd	17	0.67	0.33 – 3.33	**p = 0.006**
Molars (n = 20)	nd	4	0.33	0.33 – 0.67	0.33	12	1.0	0.33 – 3.33	**p = 0.01**
Incisors (n = 21)	nd	2	0.5	0.33 – 0.67	nd	5	0.33	0.33 – 1.33	p = 0.22

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.33 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
nd = not detected
n = number of subjects
NC = Not calculable

Table 14: Anaerobic Intensity of Bacteraemia (cfu/ml) at Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min - Max‡	Median	N^{DB}	Median	Min - Max ‡	Significance
Rubber dam	nd	6	0.33	NC	nd	16	0.83	0.33 – 10.33	**p = 0.001**
Fast drill	nd	4	0.33	NC	nd	5	0.33	0.33 – 4.67	p = 0.4
Slow drill	nd	2	0.5	0.33 – 0.67	nd	1	0.5	NC	p = 1.0
Matrix band and wedge	nd	6	0.33	NC	0.33	21	0.67	0.33 – 24.7	**p = 0.0001**
Gingival retraction cord	nd	4	0.33	0.33- 1.0	nd	15	0.67	0.33 – 4.67	**p = 0.005**
Molars (n = 20)	nd	2	0.67	0.33 – 1.0	nd	8	2.33	0.33 – 4.67	**p = 0.01**
Incisors (n = 21)	nd	2	0.33	NC	nd	7	0.33	0.33 – 3.0	**p = 0.03**

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.33 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
nd = not detected
n = number of subjects
NC = Not calculable

Total Bacterial Intensity per ml

There was a significantly greater number of cfu/ml of bacteria isolated both aerobically and anaerobically combined compared with baseline following:

1. Placement of rubber dam ($p = 0.0001$).
2. Placement of matrix band and wedge ($p = 0.0001$).
3. Placement of gingival retraction cord ($p = 0.001$). Placement of gingival retraction cord for both molar teeth ($p = 0.004$) and incisor teeth ($p = 0.05$) was significantly greater than baseline.

There was no significant difference between baseline and post-procedure intensity following use of the slow or fast drill.

(Table 15)

Table 15: Total Bacterial Intensity (cfu/ml): Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min - Max‡	Median†	N^{DB}	Median	Min - Max‡	Significance
Rubber dam	nd	12	0.17	0.17 – 0.33	0.17	22	0.33	0.17 – 8.33	**p = 0.0001**
Fast drill	nd	6	0.17	0.17 – 0.83	nd	9	0.17	0.17 – 2.67	p = 0.5
Slow drill	nd	4	0.17	0.17 – 0.33	nd	9	0.17	0.17 – 0.83	p = 0.13
Matrix band and wedge	nd	13	0.17	0.17 – 1.17	0.17	27	0.33	0.17 – 28.2	**p = 0.0001**
Gingival retraction cord	nd	9	0.17	0.17 – 0.67	0.17	23	0.33	0.17 – 2.83	**p = 0.001**
Molars (n = 20)	nd	5	0.17	0.17 – 0.67	nd	14	1.33	0.17 – 2.83	**p = 0.004**
Incisors (n = 21)	nd	4	0.17	0.17 – 0.33	nd	9	0.33	0.17 – 1.67	**p = 0.05**

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
nd = not detected
n = number of subjects

Association Between the Prevalence of Bacteraemia and the Plaque Score

There was no significant association between the aerobic, anaerobic or the combined aerobic and anaerobic prevalence of bacteraemia and the plaque scores for either the whole mouth or the individual teeth involved in each dento-gingival manipulative procedure.

Association Between the Prevalence of Bacteraemia and the Gingivitis Score

There was no significant association between the aerobic, anaerobic or the combined aerobic and anaerobic prevalence of bacteraemia and the gingivitis scores for either the whole mouth or the individual teeth involved in each dento-gingival manipulative procedure.

Association Between the Intensity of Bacteraemia and the Plaque Score

There was no significant association between the aerobic, anaerobic or the combined aerobic and anaerobic intensity of bacteraemia and the plaque scores for either the whole mouth or the individual teeth involved in each dento-gingival manipulative procedure.

Association Between the Intensity of Bacteraemia and the Gingivitis Score

There was a significant association between the anaerobic intensity of bacteraemia and whole mouth gingivitis scores (Spearman's rho = 0.42, p = 0.006) and individual gingivitis score for the teeth (Spearman's rho = 0.44, p = 0.005) involved in the placement of a gingival retraction cord.

DISCUSSION

The prevalence of bacteraemia following dental procedures such as tooth extraction, endodontic treatment, periodontal surgery and root scaling has been well documented (Coulter et al 1990; Heimdahl et al 1990; Lofthus et al 1991; Roberts et al 1997; Lucas et al 2002b). There have been few reports regarding the prevalence and intensity of bacteraemia associated with conservative dento-gingival manipulative procedures.

Each dento-gingival manipulative procedure was carried out as an isolated procedure before any planned treatment. This was to avoid confounding the results with bacteraemia from any other oral manipulation. The second blood sample was taken 30 seconds after the maximum manipulation for each dento-gingival manipulative procedure (Roberts et al 1992).

To estimate the true post-operative bacteraemia, a pre-operative or baseline blood sample was taken before each dento-gingival manipulative procedure (Heimdahl et al 1990). It has been reported that 5% (De Leo et al 1974) and 9.4% (Roberts et al 1997) of healthy children have a baseline bacteraemia unrelated to any operative procedure. In addition, a prevalence of 17% positive blood cultures was found following a dental examination in children (Roberts et al 1997). In adults a prevalence of 86% positive cultures was recorded before any procedure likely to cause a bacteraemia (Hockett et al 1977). The prevalence of bacteraemia in this study at baseline was 21.5% with an intensity ranging from 0.17 – 1.17 cfu/ml. These results are similar to those reported in other bacteraemia investigations in children (Rôberts et al 2000; Lucas et al 2002b).

The index used for recording the plaque and gingivitis scores was the modified index of O'Leary (Franco et al 1996). It is simple, reliable and does not involve probing the gingival tissues, thus avoiding transient bacteraemia (Daly et al 2001) which would confound the procedure to be investigated. This method has been shown to provide a good estimate of bacterial loading, gingival inflammation and bleeding using information from every tooth surface adjacent to the gingival margin. It has been found to be reproducible in chronically sick children (Lucas et al 1998; Sheehy et al 1999; Gelbier et al 2000; Lucas et al 2000; Harris et al 2001; Mustafa et al 2001; Sonbol et al 2001; Kidd et al 2002). Other indices which have been used include the Loe and Silness plaque index (Loe & Silness 1963) and Silness and Loe gingivitis index (Silness & Loe 1964) which give a 0–3 score for different levels of plaque and gingival inflammation. These have been used in several studies investigating bacteraemia following dental procedures (Sconyers et al 1973; Coulter et al 1990; Lofthus et al 1991; Daly et al 2001). Although the modified O'Leary index (Franco et al 1996) and the Silness and Loe (1964) and Loe and Silness indices (1963) are different

scoring methods, they have all been found to be satisfactory indicators of the amount of plaque and gingivitis in the mouth (Baldo 2003).

There was no association between plaque and gingivitis scores and the prevalence or intensity of bacteraemia in this study. This was because the plaque and gingivitis scores were low. This is in agreement with other paediatric bacteraemia investigations (Coulter et al 1990; Lucas et al 2002b). There was a significant association between the anaerobic intensity of bacteraemia and the whole mouth and individual tooth gingivitis scores for the teeth involved in the placement of a gingival retraction cord only. This suggests that overt gingival inflammation allows access of gingival crevice organisms which are predominantly anaerobic (Listgarten 1994; Wilson et al 2002) into the blood stream.

Blood volumes of 2 ml (Farrington 1973; Peterson & Peacock 1976), 5 ml (Elliott & Dunbar 1968; Coulter et al 1990), 6 ml (Lucas et al 2002b), and 8 ml (Roberts et al 2000) have been used for paediatric dental bacteraemia investigations. For adults larger volumes of 10 ml (Lineberger & De Marco 1973), 15 ml (Lofthus et al 1991), 20 ml (Lockhart 1996; Daly et al 2001), 30 ml (Khairat 1966) and 45 ml (Rogosa et al 1960) have been used. In the present study 6 ml blood samples were used to ensure comparability with recent odontogenic bacteraemia investigations in children (Lucas & Roberts 2000; Roberts et al 2000; Lucas et al 2002b).

Bacteria are assumed to be distributed through the blood as it circulates the body. Hence the size of the blood sample inoculated onto the culture medium significantly influences the number of samples determined to be positive (Bender et al 1961). The common assumption is that the magnitude of bacteraemia in infants and children is greater than that in adults and lesser volumes of blood are sufficient for culture (Welch et al 1985; Washington & Ilstrup 1986). A number of workers have recommended that the volume of blood cultured from a child patient should be directly related to their weight, the estimated total blood volume and age (Kaditis et al 1996; Kellogg et al 2000). In the investigation reported here, only children weighing more than 17.5 kg were included following discussion with the ethical committee and consultant anaesthetists.

There has been a wide variation in the time intervals for collecting blood samples following dento-gingival manipulative procedures. These range from 30 seconds following toothbrushing (Bhanji et al 2002), 2 minutes following orthodontic banding (Erverdi et al 1999), and 5 minutes (Elliott & Dunbar 1968) to 10 minutes following extractions (Burket & Burn 1937). The frequency of detection of bacteraemia varies with the time that blood is

taken after the dental procedure. Different blood sampling times in adults ranging from 45 seconds to 24 minutes following extraction of teeth have been investigated (Jokinen 1970). A higher yield of positive cultures was recorded between 0 to 45 seconds. Work with children demonstrated a clear peak in detectable bacteraemia at 30 seconds following dental extractions (Roberts et al 1992). A more recent investigation has demonstrated a peak at both 30 seconds and 1 minute post-procedure (Lucas et al 2001).

Contamination of blood cultures may occur during any stage of the process, but it is widely believed to occur during venepuncture procedures (Tilton 1982). The common explanation is that the insertion of the needle through poorly disinfected skin causes contamination of the needle by indigenous microbial flora which are inoculated into the syringe as the blood is withdrawn. As a result, strict antiseptic preparation of the venepuncture site and use of antiseptic techniques are recommended even though the quality of antiseptic procedures have not shown an effect on the contamination rate of blood cultures (Shahar et al 1990). During the present research, the first 0.5 ml of blood withdrawn through the cannula was discarded as a precaution to avoid contamination (Roberts 1997).

Several groups of workers have shown lysis filtration to be an effective method of estimating the intensity of bacteraemia following dental extractions in adults (Heimdahl et al 1985; Heimdahl et al 1990) and in children (Lucas et al 2002a). The technique of lysis filtration provides significantly greater yields from blood than broth based systems in experimental animals (Zierdt et al 1982) and bacteraemia studies (Lucas et al 2002a). Lysis filtration also enables the estimation of the intensity of bacteraemia which is not possible with broth culture techniques. Optimal bacterial yield is recovered by this technique for a number of reasons. These include dilution of blood and the use of the anticoagulant SPS (Traub & Kleber 1977; Salventi et al 1979). In addition, lysis solution neutralizes the antimicrobial components of the blood, inhibits phagocytosis of bacteria and releases intracellular organisms by lysis of phagocytes (Zierdt 1982). This allows the recovery of small numbers of bacteria by concentration of microorganisms onto the membrane filter.

There are certain limitations for detecting bacteria from blood using this technique. In the case of the present study a small volume of blood was taken in line with ethical concerns for the safety of the child. It is was necessary to define detection limits emphasizing that below a certain intensity, although bacteria may be present, they could not be successfully recovered.

Another important point to highlight is that the calculation of the total intensity of bacteraemia by adding aerobic and anerobic counts may underestimate the actual bacteraemia present. This is due to the inability of obligate anaerobes and obligate aerobes to grow in aerobic and anaerobic media respectively (Appendix 6). However, due to the fact that the majority of the bacteria isolated in this study were facultative (Chapter 4), this underestimation is very small.

The prevalence of bacteraemia reported in children following dental extractions ranges from 21% to 63% (Elliott & Dunbar 1968; Peterson & Peacock 1976; Hess et al 1983b; Roberts et al 1987; Coulter et al 1990). Dental scaling also causes bacteraemia ranging from 40% to 70% (Heimdahl et al 1990; Roberts et al 1997). In the present study, a significantly greater prevalence of bacteraemia compared to baseline was found following placement of matrix band and wedge (66%), rubber dam (54%) and gingival retraction cord (56%). This is in agreement with earlier workers who reported a significantly greater prevalence of positive blood cultures between a baseline group and placement of matrix band and wedge (32%) and rubber dam (31%) groups (Roberts et al 2000).

There was also a significantly greater intensity of bacteraemia compared to baseline following placement of matrix band and wedge, rubber dam and gingival retraction cord. This is in agreement with earlier work (Roberts et al 2000). These researchers reported a larger range in intensity following rubber dam placement (0 – 100,000 cfu/ml) and following matrix band and wedge (0 – 128 cfu/ml) compared with this study. A possible explanation for this is that these workers used the Isolator system to determine the number of colony forming units per ml. This technique requires immediate laboratory handling of the specimen or the yield of the microorganisms becomes inaccurate (Dorn et al 1979). There is no research on the prevalence and intensity of bacteraemia following placement of gingival retraction cord.

The prevalence and intensity of bacteraemia following the use of the fast and slow drill was not significantly different from baseline except for aerobically following use of the slow drill. The prevalence of positive blood cultures following use of both the fast drill (22%) and slow drill (22%) in this study are greater than those recorded by other workers, 4.3% and 12.2% respectively (Roberts et al 2000). These differences are due to variations in blood processing techniques used.

Summary

The prevalence and intensity of bacteraemia were significantly greater than baseline following placement of rubber dam, matrix band and wedge and gingival retraction cord. There was no significant difference in the prevalence and intensity of bacteraemia compared to baseline following use of the slow or fast drill.

CHAPTER 4

IDENTIFICATION OF BACTERIA ISOLATED FROM BLOOD SAMPLES USING 16S rRNA GENE SEQUENCING

INTRODUCTION

16S rRNA partial gene sequencing

The major breakthrough of rRNA sequencing techniques (Fox & Woese 1975) has heralded a new era in bacterial identification and classification. The 16S rRNA region is conserved and functionally constant (Woese 1987) and the use of RNA allows direct and rapid sequencing to be carried out by means of the enzyme reverse transcriptase (Lane et al 1985). Broad-range primers recognizing conserved regions of the 16S rRNA gene are used to amplify intervening variable sequences. The sequence data is compared with sequences of known bacteria on databases to provide a determinant of genetic relatedness. Using this method, phylogenetic trees, based on nucleotide base differences between species, are constructed and bacteria have been classified and reclassified into new genera (Olsen et al 1992). The accessibility and ease of use of bacterial genetic information has enabled the use of 16S rRNA techniques to become established for bacterial identification in clinical laboratories (Drancourt et al 2000; Woo et al 2002). This technique has been used successfully to describe the bacteria genera and species found in the human oral cavity (Tanner et al 1994; Paster et al 2001).

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a highly sensitive technique by which minute quantities of specific RNA or DNA sequences are enzymatically amplified to produce a sufficient quantity of material to reach a threshold signal for detection (Persing 1991). The fundamental basis of this technique is that each microorganism possesses a unique signature sequence in the DNA or RNA composition by which it can be identified. These target signature sequences are synthesized *in vitro* by PCR. This involves repeated replication of a given sequence of nucleotide. The agents necessary for the reactions are the single stranded oligonucleotide primers, deoxynucleotide triphophates, *Taq* polymerase and the bacterial DNA or RNA. In practice, two specific oligonucelotide primers are added so that they flank the region of interest. These are placed together in a small vial in an automated, computerized hot block or thermal cycler. The thermal cycler is able to elevate, hold and cool the temperature of the vials to allow DNA denaturation at 94-100°C followed by annealing of the primers during a cooling step. Extension of the primers by DNA synthesis occurs by the thermostable *Taq* polymerase which is purified from *Thermophilus aquaticus* (Kim et al 2002). Repetition of heat denaturation and annealing primer extension results in

the logarithmic amplification of the sequence that is located between the primers (Figure 12). The primers used for the PCR in this study were 27f and 1492r as they are ideal for PCR amplification of 16S rRNA genes (Medlin et al 1988).

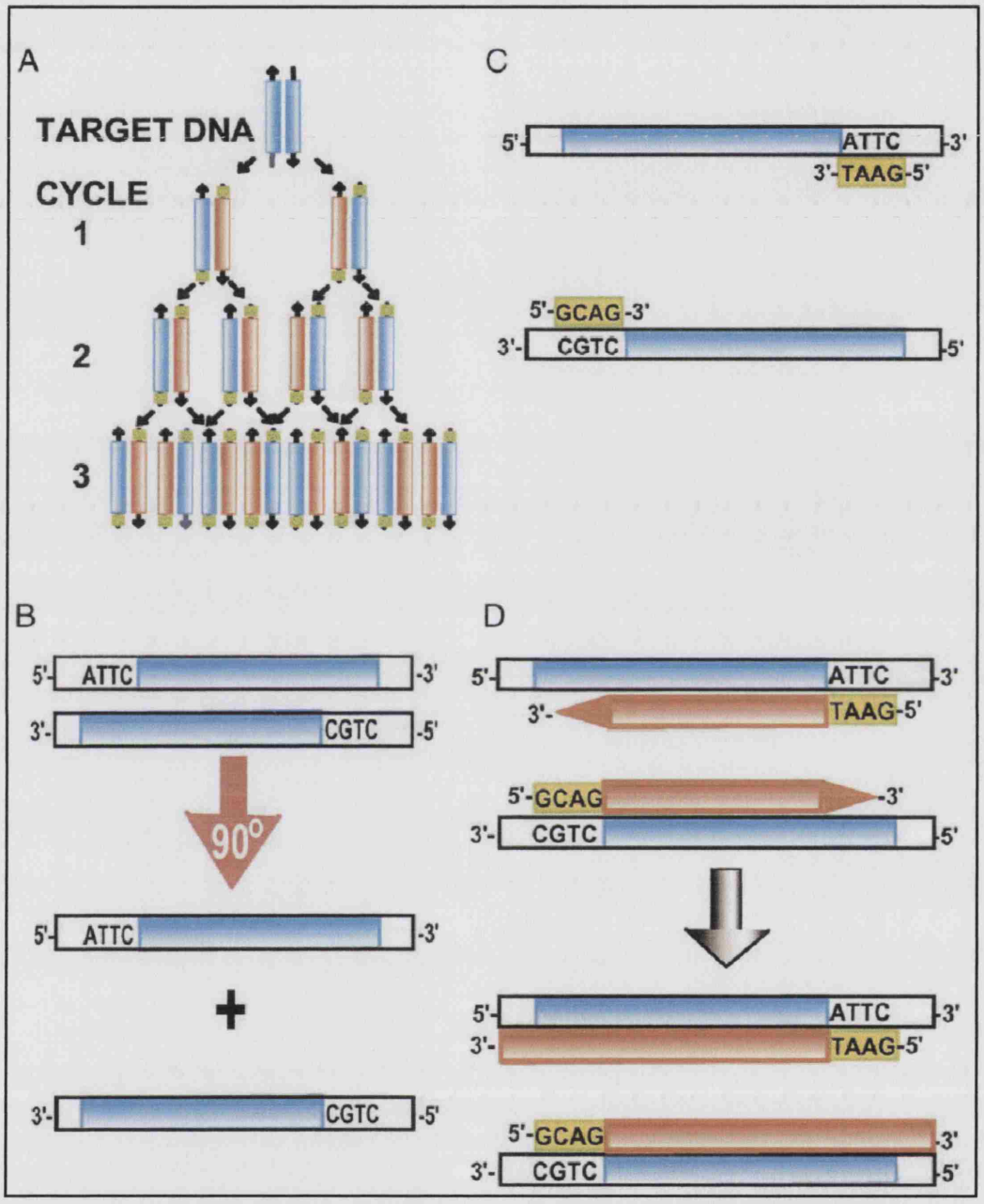


Figure 12: DNA amplification by PCR (after Kim et al 2002)

A: Target DNA (blue box) is theoretically amplified with each PCR cycle. In each cycle, newly synthesized DNA (red box) is generated from a gene-specific primer (green box).
B: Each PCR cycle begins with denaturation or separation of the target DNA.
C: Primers (green box), that mirror or complement a known portion of the target DNA, anneal to the target DNA (blue box).
D: Newly synthesized DNA (red box) is synthesized from the primer.

MATERIALS AND METHODS

Identification of Bacteria to Genus Level

Gram stain

At least one colony forming unit of bacteria of each different type of morphology was Gram-stained and subjected to further tests.

Oxygen tolerance

Bacteria isolated following anaerobic incubation were subcultured onto CBA and incubated aerobically for 24 hours.

Aerobes

Bacteria isolated following aerobic incubation were subcultured onto FAA and incubated anaerobically for 24 hours.

Anaerobes

Obligate anaerobes were identified by subculturing the isolate on FAA inoculated with a metronidazole disc (Oxoid). An area of growth inhibition around the disc indicated an obligate anaerobe (Figure 13).

Figure 13: Area of bacterial growth inhibition around a metronidazole disc

Catalase test

Growth from the centre of a colony was inoculated onto a clean glass slide. One drop of 3% hydrogen peroxide (Sigma) was added. The appearance of bubbles or effervescence indicated a positive result (Bascomb & Manafi 1998).

Oxidase test

A loopful of tetramethyl-p-phenylenediamine dihydrochloride (BDH, Poole, Dorset, UK) was inoculated into approximately 3 ml of sterile distilled water. A sterile cotton swab soaked in this solution was inoculated with bacteria from the agar plate. The development of a purple colour on the swab within 10 seconds was recorded as a positive reaction (Cowan & Steel 1993).

Coagulase test

A Staphytect Plus kit (Oxoid) was used. A drop of the commercial reagent was added to bacterial colonies smeared onto the reaction card and mixed. A positive result was indicated by the presence of agglutination within 20 seconds.

All bacteria isolated were frozen in duplicate at –80°C as follows:

1. A 1:1 ratio of Fastidious Anaerobe Broth (Oxoid) and phosphate buffered saline (PBS) tablets (Oxoid) supplemented with 10% glycerol.
2. A commercial system containing porous beads and cryopreservative (Microbank, Pro-lab Diagnostics, South Wirral, Cheshire, UK).

Speciation of bacteria

All bacterial isolates were subjected to partial sequencing of the 16S rRNA gene region (Woese 1987). Oral streptococci were further speciated by:

1. Partial sequencing of the manganese-dependant superoxide dismutase (*sodA*) gene (Poyart et al 1998) (Chapter 5)
2. Carbohydrate fermentation and enzyme hydrolysis tests (Whiley et al 1990a; Beighton et al 1991a; Beighton et al 1991b) (Chapter 5)
3. Restriction fragment length polymorphism analysis (RFLP) of the *sodA* gene (Chapter 6)

Coagulase-negative staphylococci were identified by:

1. Partial sequencing of the *sodA* gene (Poyart et al 2001)
2. API ID 32 Staph system (Biomerieux, Marcy l'Etoile, France)

(Chapter 7)

16S rRNA gene sequencing

Amplification of 16S rRNA region

Single bacterial colonies were suspended in a total reaction volume of 100 µl PCR mastermix containing the following: 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP; Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 0.2µM of each PCR primer 27f and 1492r (Genosys, Cambridge, Cambridgeshire, UK), 1 × reaction buffer [$(NH_4)_2SO_4$, Tris-HCL Tween-20; Bioline, London, UK] and 1 U of Taq polymerase (Bioline).

PCR amplification was performed in a thermal cycler (Primus, MWG-Biotech AG, Ebersberg, Germany) according to the following reaction parameters: 94°C for 5 minutes, 29 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 1.5 minutes, followed by an extension period of 72°C for 5 minutes. A negative control using sterile, nuclease-free water and a positive control of *Capnocytophagae ocheracea* template were also prepared for each reaction.

Primers used for 16S rRNA PCR Amplification and Sequencing

Primer	Sequence
27f	5' AGAGTTTGATCMTGGCTCAG
1492r	5' TACGGYTACCTTGTTACGACTT
357f	5' CTCCTACGGGAGGCAGCAG

Y=C+T, M=A+C

PCR products of approximately 1500 base pairs (bp) were assessed using gel electrophoresis in a 1% agarose gel (Amresco, Solon, OH, USA) made with Tris-acetate EDTA buffer (TAE; Eppendorf, Hamburg, Germany) containing ethidium bromide at a concentration of 0.5 µg/ml. The end well was loaded with a molecular weight marker (50-2000 bp; Amresco). Five microlitres of PCR product and 1 µl of loading buffer (Sigma) were loaded in the remaining wells. The gel was immersed in TAE buffer and subjected to a voltage difference of 100 volts to separate the fragments. Visualization of the gel was performed under ultraviolet transillumination in a Multiimage Light Cabinet (AlphaInnotech Corp., Cannock, Staffordshire, UK) and the image (Figure 14) captured by a charge coupled device (CCD) camera and manipulated using Alphaimager computer software version 5.5 (AlphaEase™, AlphaInnotech).

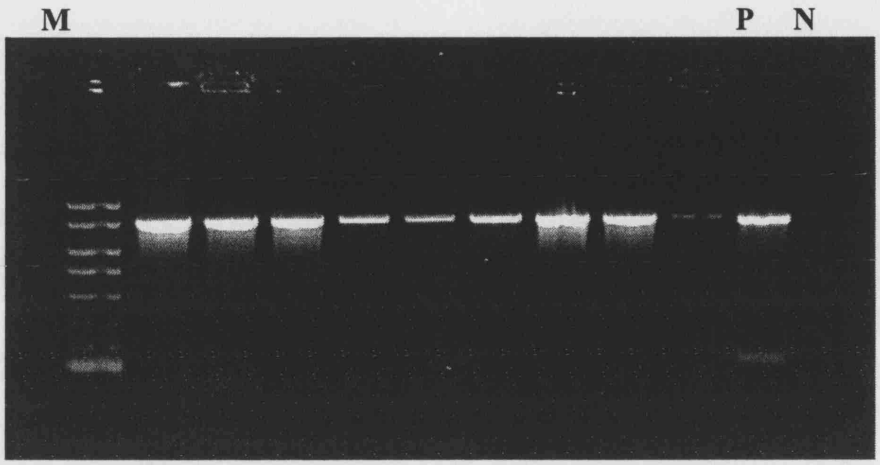


Figure 14: Gel image of 16S rRNA PCR products
(M – molecular weight marker, P – positive control, N – negative control)

16S rRNA partial gene sequencing

The PCR products were cleaned using QIAquick PCR purification kit (Qiagen, Crawley, West Sussex, UK) and stored at –20˚C until sequencing. The sequencing reaction was carried out by adding 3 µl of sterile nuclease-free water, 2 µl of ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, Cheshire, UK) diluted 1:4 in 5 × sequencing buffer (400 mM TRIS, 10 mM $MgCl_2$), 1 µl of 357f primer (5 pmol μl^{-1}; Genosys) and 1 µl of DNA template to give a final reaction volume of 7 µl. The reaction was performed in a thermal cycler (Primus) according to the following program; 99 cycles at 95˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 4 minutes.

A final clean up was performed by adding 15 µl of sterile nuclease-free water, 2 µl 3M sodium acetate and 50 µl 95% cold (–20ºC) ethanol (Anachem, Luton, Bedfordshire, UK) to the PCR tubes and then transferring the mixture to 1.5 ml eppendorf tubes. The tubes were incubated on ice for 20 minutes and then centrifuged at 14k (14,000 rpm) for 25 minutes at 4ºC. The liquid was gently removed by pipetting and washed with 250 µl of cold (–20ºC) 70% ethanol. The tubes were centrifuged again at 14k (14,000 rpm) at 4ºC for 15 minutes. The liquid was removed by pipetting and the tubes dried on a heating block at 95ºC for a few seconds with the lids removed. The DNA was re-suspended in 20 µl of Template Suppresor Reagent (TSR; Applied Biosystems), placed on the heating block at 95ºC for 2 minutes, vortexed and finally transferred to labelled 0.5 ml sequencing tubes and placed on an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems).

Computer software program Chromas (version 1.45, Queensland, Australia) was used to analyze the resulting electrophoretograms. Stringent criteria were used and only sequences with 300 or more bases, tall peaks, low background noise and a minimal number of unidentified bases (N's) were submitted to the Basic Logic Alignment Search Tool 2.2.1 (BLAST) analysis database (Altschul et al 1990; Altschul et al 1997). The matches with the highest alignment scores were accepted as final species identification (Appendix 7).

Statistical Analysis

Non-parametric statistical testing was carried out on the microbiological data (Altman 1991; Olsen 2003). McNemar's test was used to detect any difference between baseline and post-procedure samples in the proportion of positive blood cultures for each bacterial genus. The median intensity of the whole group and the median, minimum and maximum values for subjects with positive blood cultures (detectable bacteraemia) are presented. The Wilcoxon signed ranks test was used to compare baseline and post-procedure intensity for each bacterial genus.

RESULTS

A total of 721 cfu's were recovered from the membrane filters of which 67 were from baseline blood samples and 654 from post-procedure samples. There were 324 bacteria isolated aerobically and 397 anaerobically. Where possible, two isolates of each colony morphology underwent preliminary identification as described. Bacteria were frozen as representative isolates (n = 321) according to their distinctive colony morphology. Identification of the isolates using partial sequencing of the 16S rRNA gene is shown in Table 16. Eleven isolates were unrecoverable following subculture.

Table 16: Bacterial Isolates Identified by 16S rRNA Gene Sequencing

Bacteria	Baseline	Post-procedure					Total cfu's
		Rubber dam	Fast drill	Slow drill	Matrix band and wedge	Gingival retraction cord	
Streptococci	2	86	3	1	209	31	**332**
Staphylococci	34	29	11	5	34	30	**143**
Micrococci	11	2	2	1	2	9	**27**
Micrococcus luteus	11	2	2	1	2	9	27
Propionibacteria	3	4	10	2	3	6	**28**
P. acnes	3	4	10	2	3	6	28
Rothia	1	2	-	1	3	7	**14**
R. mucilaginosa	-	1	-	1	2	5	9
R. dentocariosa	1	1	-	-	1	2	5
Neisseria	-	2	-	2	4	4	**12**
N. flava-like*	-	2	-	2	1	4	9
N. pharynges	-	-	-	-	3	-	3
Actinomyces		7	-	-	74	5	**86**
A. odontolyticus	-	-	-	-	55	1	56
A. naeslundii	-	6	-	-	7	2	15
A. viscosus	-	1	-	-	3	1	5
A. georgiae	-	-	-	-	2	-	2
A. lingnae	-	-	-	-	1	-	1
A. gerencseriae	-	-	-	-	6	-	6
Actinomyces sp.	-	-	-	-	-	1	1
H. parainfluenzae	-	-	-	-	21	-	21
Aerococcus viridans	-	-	1	-	-	13	14
Abiotrophia defectiva	-	-	-	-	-	3	3
Veillionella parvula	-	-	-	-	2	-	2

* *N. mucosa, N. sicca. N. subflava, N. flava* could not be differentiated and were collectively called *N. flava*-like

Table 16: Bacterial Isolates Identified by 16S rRNA Gene Sequencing (cont.)

Bacteria	Baseline	Post-procedure					Total cfu's
		Rubber dam	Fast drill	Slow drill	Matrix band and wedge	Gingival retraction cord	
Corynebacterium	2	-	-	-	2	-	**4**
C. aurimucosum	1	-	-	-	-	-	1
C. lipophilia	-	-	-	-	1	-	1
C. coyleae	1	-	-	-	-	-	1
C. lipophilioflavum	-	-	-	-	1	-	1
Brachybacterium	3	-	-	1	1	-	**5**
B. sacelli	1	-	-	-	1	-	2
B. rhamnosum	-	-	-	1	-	-	1
B. conglomeratum	1	-	-	-	-	-	1
B. paraconglomeratum	1	-	-	-	-	-	1
Actinobacterium sp.	2	-	-	-	-	-	2
Actinobaculum sp.	-	-	-	-	-	1	1
Acinetobacter lwoffi	-	-	-	-	-	3	3
Dermabacter hominis	-	-	-	-	2	-	2
Brevibacterium celer	2	-	-	-	-	-	2
Kocuria palustris	-	-	-	-	-	1	1
Kytococcus shroeteri	-	-	-	1	-	-	1
Paenibacillus lautus	-	-	-	1	-	-	1
Bacillus cereus	-	-	-	-	-	1	1
Enterococcus faecalis	1						1
Unidentified[Δ]	6	5		1		3	15
Gram-positive rod	3	5				1	9
Gram-positive cocci (catalase-positive)	3			1		2	6

Δ Could not be recovered from frozen culture

Proportion of Each Bacterial Genus as a Percentage of the Total Number of Bacteria Isolated: Baseline and Following Each Dento-Gingival Manipulative Procedure

Baseline

Sixty-seven bacterial colonies were identified from baseline blood samples of the five procedure groups investigated. Thirty-four colonies (50.7%) were *Staphylococcus* spp., two (2.9%) were *Streptococcus* spp., 11 (16.4%) were *Micrococcus* spp., two (4.5%) were *Propionibacterium* spp. and one (1.5%) was a *Rothia* sp.

Following All Dento-Gingival Manipulative Procedures

Six hundred and fifty-four bacterial colonies were identified from post-procedure blood samples. Three hundred and thirty bacterial colonies (50.5%) were *Streptococcus* spp., 110 (16.8%) were *Staphylococcus* spp., 86 (13.1%) were *Actinomyces* spp., 25 (3.8%) were *Propionibacterium* spp., 21 (3.2%) were *Haemophilus* spp., 16 (2.4%) were *Micrococcus* spp., 13 (2%) were *Rothia* spp. and 12 (1.8%) were *Neisseria* spp.

One hundred and thirty-seven bacterial colonies (20.9%) were isolated following rubber dam placement. Twenty-seven (4.1%) were isolated following use of the fast drill and 16 (2.5%) following use of the slow drill. Three hundred and fifty-seven bacterial colonies (54.6%) were isolated following matrix band and wedge placement and 117 (17.9%) following gingival retraction cord placement. The proportion of each bacterial genus as a percentage of the total number of bacteria isolated following each individual dento-gingival manipulative procedure is shown in Table 17.

Table 17: Proportion of Each Bacterial Genus as a Percentage of the Total Number of Bacteria Isolated Following Each Dento-Gingival Manipulative Procedure

Bacteria	Rubber dam (n = 137)	Fast drill (n = 27)	Slow drill (n = 16)	Matrix band and wedge (n = 357)	Gingival retraction cord (n = 117)
Streptococcus spp.	62.8%	11.1%	6.3%	58.5%	26.5%
Staphylococcus spp.	21.2%	40.7%	31.3%	9.5%	25.6%
Actinomyces spp.	5.1%	0	-	20.7%	4.3%
Haemophilus spp.	-	-	-	5.9%	-
Micrococcus spp	1.5%	7.4%	6.3%	0.5%	7.7%
Propionibacterium spp.	2.9%	37%	12.5%	0.5%	5.1%
Rothia spp.	1.5%	0	6.3%	0.8%	6%
Neisseria spp.	1.5%	0	6.3%	1.1%	3.4%
Aerococcus spp.	-	-	-	-	11.1%

n = number of isolates

Isolation Frequency and Intensity of Each Bacterial Genus

There was a significantly greater isolation frequency post-procedure compared to baseline for the following bacteria:

1. *Streptococcus* spp: baseline 1% and post-procedure 18.5% (p = 0.0001)
2. *Staphylococcus* spp.: baseline 14% and post-procedure 27% (p = 0.001)
3. *Actinomyces* spp.: baseline not detected and post-procedure 9% (p = 0.0001)
4. *Rothia* spp.: baseline 0.5% and post-procedure 4% (p = 0.02)
5. *Propionibacterium* spp.: baseline 1.5% and post-procedure 5.9% (p = 0.02)

(Table 18)

Table 18: Isolation Frequency of Each Bacterial Species: Baseline and Following All Dento-Gingival Manipulative Procedures

	Baseline (n = 205)		Post-procedure (n = 205)		
	%	Number	%	Number	Sig
***Streptococcus* spp.**	1	2	18.5	38	**p = 0.0001**
***Staphylococcus* spp.**	14	29	27	55	**p = 0.001**
***Actinomyces* spp.**	nd	nd	9	18	**p = 0.0001**
***Rothia* spp.**	0.5	1	4	8	**p = 0.02**
***Neisseria* spp.**	nd	nd	2.4	5	p = 0.13
***Micrococcus* spp.**	3.9	8	4.9	10	p = 0.82
***Propionibacterium* spp.**	1.5	3	5.9	12	**p = 0.02**
***Haemophilus* spp.**	nd	nd	1.0	2	p = 0.22

n = number of subjects
nd = not detected (< 0.17 cfu/ml)
Sig = statistical significance

There was a significantly greater number of cfu/ml from post-procedure blood samples compared to baseline for the following bacteria:

1. *Streptococcus* spp. (p = 0.0001)
2. *Staphylococcus* spp. (p = 0.0001)
3. *Actinomyces* spp. (p = 0.0001)
4. *Rothia* spp. (p = 0.03)
5. *Neisseria* spp. (p = 0.04)
6. *Propionibacterium* spp. (p = 0.02)

(Table 19)

Table 19: Intensity (cfu/ml) of Each Bacterial Species: Baseline and Following All Dento-Gingival Manipulative Procedures

	Baseline (n = 205)				Post-procedure (n = 205)				
	Median†	N^{DB}	Median	Min-Max‡	Median†	N^{DB}	Median	Min-Max‡	Sig
***Streptococcus* spp.**	nd	2	0.17	NC	nd	38	0.42	0.17 – 19.2	**p = 0.0001**
***Staphylococcus* spp.**	nd	29	0.17	0.17 – 0.67	nd	55	0.17	0.17 – 2.0	**p = 0.0001**
***Actinomyces* spp.**	nd	0	-	-	nd	18	0.17	0.17 – 8.83	**p = 0.0001**
***Rothia* spp.**	nd	1	0.17	NC	nd	8	0.17	0.17 – 0.67	**p = 0.03**
***Neisseria* spp.**	nd	0	-	-	nd	5	0.33	0.17 – 0.67	**p = 0.04**
***Micrococcus* spp.**	nd	8	0.17	0.17 – 0.5	nd	10	0.17	0.17 – 1.0	p = 0.71
***Propionibacterium* spp.**	nd	3	0.17	0.17 – 0.33	nd	12	0.17	0.17 – 1.5	**p = 0.02**
***Haemophilus* spp.**	nd	0	-	-	nd	5	0.17	0.17 – 3.33	p = 0.07

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia

nd = not detected
n = number of subjects
NC = not calculable

Isolation Frequency and Intensity of Bacterial Species Following Each Dento-Gingival Manipulative Procedure

***Streptococcus* spp.**

The isolation frequency of *Streptococcus* spp. isolated following each of the dento-gingival manipulative procedures is shown in Table 20. There was a significantly greater isolation frequency of *Streptococcus* spp. post-procedure compared to baseline after the following procedures:

1. Placement of rubber dam: baseline not detected and post-procedure 27% (p = 0.006)
2. Placement of matrix band and wedge: baseline 2% and post-procedure 34% (p = 0.004)
3. Placement of gingival retraction cord: baseline 2% and post-procedure 24% (p = 0.01)

Table 20: Isolation Frequency of *Streptococcus* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure	Baseline		Post-procedure		
(n = 41)	%	Number	%	Number	Sig
Rubber dam	nd	nd	27	11	**p = 0.006**
Fast drill	nd	nd	5	2	p = 1.0
Slow drill	nd	nd	2	1	p = 1.0
Matrix band and wedge	2	1	34	14	**p = 0.004**
Gingival retraction cord	2	1	24	10	**p = 0.01**

n = number of subjects
nd = not detected (< 0.17 cfu/ml)
Sig = statistical significance

The intensity of *Streptococcus* spp. isolated following each dento-gingival manipulative procedure is shown in Table 21. There was a significantly greater number of cfu/ml of *Streptococcus* spp. compared with baseline after the following dento-gingival manipulative procedures:

1. Placement of rubber dam (p = 0.003)
2. Placement of matrix band and wedge (p = 0.002)
3. Placement of gingival retraction cord (p = 0.006)

Table 21: Intensity (cfu/ml) of *Streptococcus* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min-Max‡	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	nd	0	-	-	nd	11	0.67	0.17 – 7.17	**p = 0.003**
Fast drill	nd	0	-	-	nd	2	0.25	0.17 – 0.33	p = 0.18
Slow drill	nd	0	-	-	nd	1	0.17	NC	p = 0.32
Matrix band and wedge	nd	1	0.17	NC	nd	14	0.42	0.17 – 19.2	**p = 0.002**
Gingival retraction cord	nd	1	0.17	NC	nd	10	0.42	0.17 – 1.67	**p = 0.006**

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia

nd = not detected
n = number of subjects
NC = not calculable

***Staphylococcus* spp.**

The isolation frequency of *Staphylococcus* spp. isolated following each of the dento-gingival manipulative procedures is shown in Table 22. There was a significantly greater isolation frequency of *Staphylococcus* spp. post-procedure compared to baseline after the following procedures:

1. Placement of matrix band and wedge: baseline 19% and post-procedure 41% (p = 0.02)
2. Placement of gingival retraction cord: baseline 15% and post-procedure 39% (p = 0.03)

Table 22: Isolation Frequency of *Staphylococcus* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline		Post-procedure		
	%	Number	%	Number	Sig
Rubber dam	24	10	34	14	p = 0.34
Fast drill	7	3	12	5	p = 0.73
Slow drill	5	2	7	3	p = 1.0
Matrix band and wedge	19	8	41	17	**p = 0.02**
Gingival retraction cord	15	6	39	16	**p = 0.03**

n = number of subjects
Sig = statistical significance

The intensity of *Staphylococcus* spp. isolated following each dento-gingival manipulative procedure is shown in Table 23. There was a significantly greater number of cfu/ml of *Staphylococcus* spp. compared with baseline after the following dento-gingival manipulative procedures:

1. Placement of rubber dam (p = 0.04)
2. Placement of matrix band and wedge (p = 0.02)
3. Placement of gingival retraction cord (p = 0.02)

Table 23: Intensity (cfu/ml) of *Staphylococcus* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min-Max‡	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	nd	10	0.17	0.17 – 0.33	nd	14	0.25	0.17 – 1.17	**p = 0.04**
Fast drill	nd	3	0.17	NC	nd	5	0.17	0.17 – 1.17	p = 0.37
Slow drill	nd	2	0.17	NC	nd	3	0.33	0.17 – 0.33	p = 0.33
Matrix band and wedge	nd	8	0.17	0.17 – 0.67	nd	17	0.17	0.17 – 0.83	**p = 0.02**
Gingival retraction cord	nd	6	0.17	0.17 – 0.33	nd	16	0.17	0.17 – 2.0	**p = 0.02**

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
nd = not detected
n = number of subjects
NC = not calculable

***Actinomyces* spp.**

The isolation frequency of *Actinomyces* spp. isolated following each of the dento-gingival manipulative procedures is shown in Table 24. There was a significantly greater isolation frequency of *Actinomyces* spp. post-procedure compared to baseline following placement of a matrix band and wedge ($p = 0.002$).

Table 24: Isolation Frequency of *Actinomyces* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline		Post-procedure		
	%	Number	%	Number	Sig
Rubber dam	nd	nd	10	4	p = 0.38
Fast drill	nd	nd	nd	nd	-
Slow drill	nd	nd	nd	nd	-
Matrix band and wedge	nd	nd	24	10	**p = 0.002**
Gingival retraction cord	nd	nd	10	4	p = 0.22

n = number of subjects
nd = not detected (< 0.17 cfu/ml)
Sig = statistical significance

The intensity of *Actinomyces* spp. isolated following each dento-gingival manipulative procedure is shown in Table 25. There was a significantly greater number of cfu/ml of *Actinomyces* spp. compared with baseline following placement of a matrix band and wedge ($p = 0.004$). There was a trend towards significance for the intensity of *Actinomyces* spp. following placement of rubber dam ($p = 0.06$) and gingival retraction cord ($p = 0.06$).

Table 25: Intensity (cfu/ml) of *Actinomyces* spp: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline				Post-procedure				
	Median†	N^{DB}	Median	Min-Max‡	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	nd	0	-	-	nd	4	0.17	0.17 – 0.67	p = 0.06
Fast drill	nd	0	-	-	nd	0	-	-	-
Slow drill	nd	0	-	-	nd	0	-	-	-
Matrix band and wedge	nd	0	-	-	nd	10	0.25	0.17 – 8.83	**p = 0.004**
Gingival retraction cord	nd	0	-	-	nd	4	0.17	0.17 – 0.33	p = 0.06

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
nd = not detected
n = number of subjects

Other Bacteria

The isolation frequency and intensity of *Rothia* spp., *Neisseria* spp., *Micrococcus* spp., *Propionibacterium* spp. and *Haemophilus* spp. isolated following each of the dento-gingival manipulative procedures is shown in Tables 26–30, respectively. No significant difference in post-procedure isolation frequency or intensity compared to baseline following each dento-gingival manipulative procedure was found for these bacteria.

Table 26: Isolation Frequency and Intensity (cfu/ml) of *Rothia* spp: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline					Post-procedure					
	%	Median†	N^{DB}	Median	Min-Max‡	%	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	2.4	nd	1	0.17	NC	7.3	nd	3	0.17	NC	p = 0.71
Fast drill	nd	-	0	-	-	nd	-	0	-	-	-
Slow drill	nd	-	0	-	-	1.2	nd	1	0.17	NC	p = 0.32
Matrix band and wedge	nd	-	0	-	-	4.8	nd	2	0.25	0.17 – 0.33	p = 0.18
Gingival retraction cord	nd	-	0	-	-	7.2	nd	3	0.33	0.17 – 0.67	p = 0.11

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia

% = Isolation Frequency
n = number of subjects
nd = not detected

NC = not calculable

Table 27: Isolation Frequency and Intensity (cfu/ml) of *Neisseria* spp: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline					Post-procedure					
	%	Median†	N^{DB}	Median	Min-Max‡	%	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	nd	-	-	-	-	2.4	nd	1	0.17	NC	p = 0.32
Fast drill	nd	-	-	-	-	nd	-	0		-	-
Slow drill	nd	-	-	-	-	2.4	nd	1	0.17	-	p = 0.32
Matrix band and wedge	nd	-	-	-	-	4.8	nd	2	0.33	0.17 – 0.67	P = 0.18
Gingival retraction cord	nd	-	-	-	-	2.4	nd	1	0.17	-	p = 0.32

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia

% = Isolation Frequency
n = number of subjects
nd = not detected

NC – not calculable

Table 28: Isolation Frequency and Intensity (cfu/ml) of *Micrococcus* spp: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline					Post-procedure					
	%	Median†	N^{DB}	Median	Min-Max‡	%	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	nd	-	0	-	-	4.8	nd	2	0.17	NC	p = 0.16
Fast drill	2.4	nd	1	0.17	NC	4.8	nd	2	0.17	NC	p = 1.0
Slow drill	nd	-	0	-	-	4.8	nd	2	0.25	0.17	p = 0.18
Matrix band and wedge	12.2	nd	5	0.17	0.17 – 1.0	nd	nd	0	-	-	p = 0.34
Gingival retraction cord	4.8	nd	2	0.17	NC	9.7	nd	4	0.17	0.17 – 1.0	p = 0.32

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
% = Isolation Frequency
n = number of subjects
nd = not detected
NC = not calculable

Table 29: Isolation Frequency and Intensity (cfu/ml) of *Propionibacterium* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline					Post-procedure					
	%	Median†	N^{DB}	Median	Min-Max‡	%	Median†	N^{DB}	Median	Min-Max‡	Sig
Rubber dam	2.4	nd	1	0.17	NC	7.3	nd	3	0.17	0.17 – 0.33	p = 0.1
Fast drill	nd	-	0	-	-	4.8	nd	2	0.83	0.17 – 1.5	p = 0.18
Slow drill	2.4	nd	1	0.17	NC	4.8	nd	2	0.17	NC	p = 1.0
Matrix band and wedge	nd	-	0	-	-	7.3	nd	3	0.17	NC	p = 0.08
Gingival retraction cord	2.4	nd	1	0.17	NC	4.8	nd	2	0.42	0.33 – 0.5	p = 0.3

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia
% = Isolation Frequency
n = number of subjects
nd = not detected
NC = not calculable

Table 30: Isolation Frequency and Intensity (cfu/ml) of *Haemophilus* spp.: Baseline and Following Each Dento-Gingival Manipulative Procedure

Procedure (n = 41)	Baseline					Post-procedure					
	%	Median†	N^{DB}	Median	Min-Max ‡	%	Median†	N^{DB}	Median	Min-Max ‡	Sig
Rubber dam	nd	-	0	-	-	nd	-	0	-	-	-
Fast drill	nd	-	0	-	-	nd	-	0	-	-	-
Slow drill	nd	-	0	-	-	nd	-	0	-	-	-
Matrix band and wedge	nd	-	0	-	-	4.8	nd	2	0.17	0.17 – 3.33	p = 0.1
Gingival retraction cord	nd	-	0	-	-	nd	-	0	-	-	-

†Median for all subjects (n = 41)
N^{DB} = number of subjects with a detectable bacteraemia (≥ 0.17 cfu/ml)
‡Median and min-max for subjects with a detectable bacteraemia

% = Isolation Frequency
n = number of subjects
nd = not detected

DISCUSSION

The bacteria isolated from blood cultures following dento-gingival manipulative procedures in this study are similar to those reported by other workers (Heimdahl et al 1990; Debelian et al 1995; Roberts et al 1997; Roberts et al 2000; Lucas et al 2002b). Bacterial isolates were initially identified using 16S rRNA partial gene sequencing. The 16S rRNA gene is present in all bacteria and is a universal target for bacterial identification. Its sequences are conserved sufficiently so that regions of homology can be clearly established. It is sufficiently large that the information content is adequate for making phylogenetic inferences (Olsen & Woese 1993; Tanner et al 1994). It is the most widely accepted gene used for bacterial classification and identification (Weisburg et al 1991; Olsen & Woese 1993).

Streptococcus and *Staphylococcus* spp. isolated in this study showed low discrimination in 16S rRNA sequences between the species with identical alignment scores and expect values (e-values) when analysed by BLAST (Altschul et al 1990; Altschul et al 1997) and could not be accurately identified to species level. This is in agreement with other workers who reported lack of discrimination of the 16S rRNA genetic target for streptococci and CNS (Poyart et al 2001; Bosshard et al 2004). At close relatedness, 16S rRNA has been found to have low discrimination between species (Fox et al 1992). A more discriminatory genetic target, namely the superoxide dismutase A gene, was necessary for species identification of oral streptococci (Chapter 5) and CNS (Chapter 7).

The largest group of bacteria isolated from post-procedure blood samples were *Streptococcus* spp., 51%. Only two streptococcal isolates were recovered, each from a baseline blood sample. This is in agreement with previous bacteraemia investigations in children (Roberts et al 1998b; Lucas & Roberts 2000; Roberts et al 2000). There was a significantly greater isolation frequency and intensity of *Streptococcus* spp. compared to baseline following rubber dam placement, matrix band and wedge placement and gingival retraction cord placement. Sixty-three percent of the *Streptococcus* spp. isolated were following placement of a matrix band and wedge.

Other catalase-negative Gram-positive cocci isolated included *Abiotrophia defectiva* and *Aerococcus viridans*. *Abiotrophia* spp. have been isolated from subgingival plaque (Paster et al 2001) and have been recently isolated from blood cultures following dental procedures in children (Lockhart et al 2004). They are rare but important causes of IE in children (Chang et al 2002; Raff & Gray 2004) with dental manipulation identified as the source of infection in several case reports (Whyman & MacFadyen 1994; Chang et al 2002; Raff & Gray 2004).

Aerococcus viridans endocarditis and bacteraemia in immunocompromised patients have also been reported (Augustine et al 1994; Uh et al 2002).

Sixty-seven isolates were identified from baseline blood samples of which 34 (51%) were CNS. This is similar to data reported by other researchers where CNS comprised 75% of baseline isolates (Roberts et al 2000; Lucas et al 2002b). Most of the CNS spp. (77%) in this study were isolated following dento-gingival manipulation which supports the concept that oral staphylococci may cause transient odontogenic bacteraemia. There was a significantly greater intensity of *Staphylococcus* spp. compared to baseline following rubber dam placement, matrix band and wedge placement and gingival retraction cord placement. Researchers investigating the relationship between age and microorganisms in patients with periodontitis found that young individuals (10-14 years) harboured the greatest number of subgingival staphylococci (Slots et al 1990). This may help to explain the large proportion of staphylococci isolated from children in the present study and other investigations in children (Roberts et al 1997; Lucas & Roberts 2000; Lucas et al 2002b).

Actinomyces spp. comprise between 40% and 90% of the cultivable flora on approximal tooth surfaces in children (Babaahmady et al 1997). Thirteen percent of the bacteria isolated from post-procedure blood samples in this study were *Actinomyces* spp. They have been isolated from blood cultures following orthodontic procedures (Lucas et al 2002b), dental extractions (Otten et al 1987; Coulter et al 1990; Heimdahl et al 1990), dental scaling (Heimdahl et al 1990) and endodontics (Debelian et al 1995). The species isolated in the present study, *A. naeslundii*, *A. odontolyticus*, *A. viscosus*, *A. georgiae* and *A. gerencseriae*, are similar to *Actinomyces* spp. isolated from the oral cavity of children by other workers (Sarkonen et al 2000; Kamma et al 2000; Tanner et al 2002). *A. odontolyticus* was the most frequently isolated species in the present study and also from the oral mucosa of infants up to 2 years (Sarkonen et al 2000) and in the dental plaque of adults (Liljemark et al 1993).

A. naeslundii was the second most common *Actinomyces* spp. isolated in this study. It was found to be the most common *Actinomyces* spp. isolated from the subgingival crevice of a different group of children with mixed dentition (Kamma et al 2000). The newly described *A. lingnae* (Clarridge & Zhang 2002) was isolated from one patient following matrix band and wedge placement. IE due to *Actinomyces* spp. is rare although there have been three reports of IE caused by *A. viscosus* (Gutschik 1976; Hamed 1998; Mardis & Many 2001). In the most recent report, the patient's teeth had been 'cleaned' one day before admission (Mardis & Many 2001).

There were 14 isolates of *Rothia* spp., of which 13 were from post-procedure blood samples. *Rothia mucilaginosa* (formerly *Stomatococcus mucilaginosus, Micrococcus mucilaginosus*) is a member of the normal oral flora (Ruoff 2002). It has been described as an opportunistic organism in cases of IE (Coudron et al 1987; Relman et al 1987; Pinsky et al 1989), infections in neutropenic patients (McWhinney et al 1992) and meningitis and peritonitis (Ascher et al 1991; Hopkins et al 1992). The oral cavity was suggested as the source of *R. mucilaginosa* septicaemia in a patient following isolation from oral mucosal ulcerations (Lemozy et al 1990). *R. mucilaginosa* (described as *Stomatococcus*) was recently isolated from blood cultures following orthodontic treatment procedures (Lucas et al 2002b). *R. mucilaginosa* is more likely to be misidentified as *Staphylococcus* than any other organism (Coudron et al 1987). If the isolate fails to produce catalase, it is sometimes identified as *Streptococcus* spp. (Rubin et al 1978).

Rothia dentocariosa (formerly *Actinomyces dentocariosus*, *Nocardia dentocariosus* or *Nocardia salivae*) is known to colonize supragingival plaque and is found in saliva (Ishikawa 1980). Since 1975, 19 cases of *R. dentocariosa* IE have been reported. Three patients were reported to have caries (Ruben 1993; Binder et al 1997; Llopis & Carratala 2000), two had periodontal disease (Binder et al 1997; Llopis & Carratala 2000), one had a fractured tooth (Broeren & Peel 1984), two had undergone dental treatment (Schafer et al 1979; Nguyen et al 2000) and one other patient had a residual molar root prior to developing IE (Isaacson & Grenko 1988). *R. dentocariosa* has recently been reported as a cause of a complicated case of IE following orthodontic treatment (Boudewijns et al 2003). *R. dentocariosa* has not been isolated from blood cultures following dental procedures prior to this investigation. This may be due to inaccurate identification using biochemical tests (von Graevenitz 2004).

A total of 28 *Propionibacterium* spp. were isolated, 25 of which (89%) were following post-procedure blood samples. They have been frequently regarded as culture contaminants (Koneman et al 1997), although *P. acnes* contamination is reported to occur in less than 3% of all blood specimens when the skin over the venepuncture site is carefully prepared (Charfreitag et al 1988). Although *Propionibacterium* spp. are usually of low level virulence, they are associated with serious infections such as IE (Lewis & Abramson 1980; Günthard et al 1994), brain abscesses (Mathisen et al 1984) and osteomyelitis (Brook & Frazier 1993). *Propionibacterium* spp. have been isolated from blood following dental extractions (Okabe et al 1995; Roberts et al 1998b) and endodontic treatment (Debelian et al 1995). Workers established the oral cavity as the source of *P. acnes* bacteraemia in patients following endodontic treatment (Debelian et al 1992). Biochemical profiles, antibiotic susceptibility

and electrophoresis of soluble proteins revealed that *P. acnes* isolated from the root canal and blood samples were identical within the same patient, but varied between patients.

Twelve isolates of *Neisseria* spp. were recovered from post-procedure blood samples in this study. *N. sicca, N. sublava, N. flava* and *N. mucosa* could not be differentiated by 16S rRNA gene sequencing and were collectively called *N. flava*-like. Phylogenetic research and DNA-DNA hybridization have demonstrated that *N. sicca, N. subflava, N. perflava, N. flava* and *N. mucosa* are closely related (Hoke & Vedros 1982; Tanner et al 1994). *Neisseria* spp. are known members of the oral flora and have been detected in subgingival plaque (Paster et al 2001) and 29-48% of approximal tooth surfaces in children (Babaahmady et al 1997). They have been isolated from blood following dental extractions (Roberts et al 1998b; Lucas et al 2002a), toothbrushing and polishing of teeth (Lucas & Roberts 2000) and conservative dental procedures (Roberts et al 2000). IE caused by *Neisseria* spp. is rare, although a number of cases have been reported in patients with dental abscesses (Lopez-Velez et al 1994), dental caries (Thornhill-Joynes & Li 1985), following dental procedures (Wong & Janda 1992), periodontal surgery (Vernaleo et al 1992) and tongue piercing (Tronel et al 2001).

In the present investigation *Haemophilus parainfluenzae* was isolated from two patients following placement of a matrix band and wedge. It has been isolated from blood cultures following dental extractions (Coulter et al 1990; Roberts et al 1998b) and scaling (Heimdahl et al 1990). *H. parainfluenzae* is the most common bacteria among HACEK organisms causing IE in adults (Das et al 1997) and children (Feder et al 2003). Recently, it has been recovered from the subgingival crevice and identified using 16S rRNA partial gene sequencing (Kroes et al 1999).

Fifty-nine percent of *Micrococcus* spp. isolated were following dento-gingival manipulation. *Micrococcus* spp. that colonize the skin, mucosa and orophaynx are considered to be normal commensals (Tanner et al 1994). They have been recognized as causes of IE (Seifert et al 1995) and as opportunistic pathogens in immunocompromised patients, causing pneumonia, septicaemia and peritonitis (Souhami et al 1979). They have been isolated from blood following dental extractions (Heimdahl et al 1985; Okabe et al 1995), endodontics (Heimdahl et al 1990), orthodontic treatment (Lucas et al 2002b) and conservative dental procedures (Roberts et al 2000). There was no significant difference in *Micrococcus* spp. isolation from post-procedure blood samples compared to baseline, suggesting that these organisms are common causes of 'everyday' transient bacteraemia.

Only four isolates of *Corynebacterium* spp. were recovered in this study 2 of which were following matrix band and wedge placement. Recently, *Corynebacterium* spp. were isolated from 9% of pus specimens from patients with dento-alveolar infections (Kuriyama et al 2002). They have been isolated from blood samples following dental extractions (Heimdahl et al 1985; Coulter et al 1990; Okabe et al 1995; Lockhart et al 2004) and dental scaling (Lucas & Roberts 2000). *Corynebacterium* spp. are rare causes of IE, although several aggressive and complicated cases have been reported (Tiley et al 1993; Daniels et al 2003).

Veillonella spp. have been isolated following dental extractions (Heimdahl et al 1990; Okabe et al 1995), scaling (Heimdahl et al 1990), conservative dental treatment (Roberts et al 2000) and toothbrushing (Lucas & Roberts 2000). One isolate of *V. parvula* was recovered from a patient following matrix band and wedge placement in the present study. *V. parvula* represented 7.3% of bacterial species from subgingival plaque samples in children with mixed dentition (Kamma et al 2000). Five cases of IE due *Veillonella* spp. have been reported (Houston et al 1997).

Summary

The most frequently isolated species following dento-gingival manipulation were *Streptococcus* spp. (51%), *Staphylococcus* spp. (17%) and *Actinomyces* spp. (13%). This is in agreement with other workers (Heimdahl et al 1990; Lucas & Roberts 2000; Roberts et al 2000; Lockhart et al 2004). They also showed a significantly greater intensity (cfu/ml) following dental manipulation compared to baseline.

CHAPTER 5

IDENTIFICATION OF STREPTOCOCCI TO SPECIES LEVEL

INTRODUCTION

Streptococci can be differentiated and classified by a combination of patterns of haemolysis on blood agar plates, antigenic composition, growth characteristics, biochemical reactions and more recently, genetic analysis (Schleifer & Kilpper-Balz 1987; Kilian et al 1989; Beighton et al 1991a). In the early 1970's a short set of biochemical tests was devised which identified most oral streptococci. These tests included acid production from mannitol and sorbitol, formation of hydrogen peroxide, hydrolysis of aesculin and arginine, acetoin production from glucose and formation of dextran or levan from sucrose (Colman & Williams 1972). A further scheme identified oral streptococci by the detection of a range of preformed glycosidase activities with chromogenic substrates, sialidase activity using fluorogenic substrates, IgA1 protease production, acid production from several carbohydrates and hydrolysis of a range of conventional substrates (Kilian et al 1989). More recently, the combination of preformed glycosidic enzyme activities combined with conventional biochemical tests have been used with fluorogenic substrates to increase sensitivity and rapidity (Beighton et al 1991a).

In clinical laboratories, streptococci are identified using phenotypic tests such as those developed for the API ID 32 Strep system (Biomerieux). The inherent problems with phenotypic tests are that not all strains within a given species may be positive for a common trait (Kilian et al 1989; Beighton et al 1991a) and that the same species may exhibit biochemical variability (Hillman et al 1989; Tardif et al 1989). In addition, commercially available systems do not have the profiles for all the species in their databases so that some identification generated by these systems are not ideal.

Nucleic acid based technology, such as amplification of selected DNA targets (Rudney & Larson 1994) and DNA hybridization (Whiley & Hardie 1989; Adnan et al 1993) have been developed and are used to complement and improve the identification of oral streptococci. 16S rRNA gene sequencing has been used to show relationships between many streptococcal species (Whiley et al 1990b; Kawamura et al 1995b). The sequence identities of the 16S rRNA genes for the type strains of *S. mitis*, *S. oralis* and *S. pneumoniae* showed greater than 99% similarity (Kawamura et al 1995b), which does not allow for sequence variability that occurs in many strains. Recently, some genetic methods for the identification of streptococci have been proposed by other researchers. These include species-specific PCR primers based on the *ddl* gene (Garnier et al 1997) and comparative analysis of the partial sequences of the *sodA* gene (Poyart et al 1998). Researchers have found sequencing the *sodA* gene to be a reliable and

practical method for accurate identification of oral streptococci, including the closely related mitis group (Kawamura et al 1999; Poyart et al 2002).

The *sodA* gene encodes the manganese-dependent superoxide dismutase enzyme. Superoxide dismutase (Sod) represents one of the major defence mechanisms of cells by impairing oxygen dependent mechanisms which are involved in the killing of bacteria by phagocytes (Pesci et al 1994). It converts superoxide anions (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The hydrogen peroxide is in turn metabolized by catalases and peroxidases (Bannister et al 1987). The enzyme is classified into three types depending on the metal cofactor utilized: copper/zinc (Cu/Zn-Sod), manganese (Mn-Sod) and iron (Fe-Sod). Mn-Sods are primarily found in prokaryotes and the mitochondria of eukaryotes; Fe-Sods in prokaryotes and in chloroplasts of eukaryotes and Cu/Zn-Sods in eukaryotes (Smith & Doolittle 1992). PCR and sequencing of the *sodA* gene encoding the manganese-dependent superoxide dismutase enzyme has been recently developed (Poyart et al 1995). An internal fragment representing approximately 85% of the *sodA* gene is amplified using degenerate primers in a polymerase chain reaction.

MATERIALS AND METHODS

Sequencing of the *sodA* Gene

Optimization of sodA PCR Parameters

Single bacterial colonies were suspended in a total reaction volume of 50 µl of PCR master mix. The PCR master mix contained: 0.6 µM of each primer, D1 and D2 (Table 31), 0.4 M dNTPs (dATP, dCTP, dGTP, dTTP; Promega), 2.5 mM $MgCl_2$, 1 × reaction buffer and 1 U Taq Polymerase (Bioline). A negative control using sterile, nuclease-free water was prepared for each reaction. PCR amplification was performed in a thermal cycler (Primus) according to the following conditions: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 37°C for 90 seconds, 72°C for 90 seconds, followed by an extension period of 72°C for 10 minutes.

Table 31 : Primers used for *sodA* PCR Amplification and Sequencing

Primer	Sequence
D1	5'-CCITAYICITAYGAYGCIYTIGARCC
D2	5- ARRTARTAIGCRTGYTCCCAIACRTC

R=A+G, Y=C+T, I=Inosine

PCR products (approximately 500 bp) were assessed using gel electrophoresis in a 1% agarose gel (Amresco) made with TAE buffer (Eppendorf) containing ethidium bromide at a concentration of 0.5 µg/ml. The end well was loaded with a molecular weight marker (50-2000 bp; Amresco). Five microlitres of PCR product and 1 µl of loading buffer (Sigma) were loaded in the remaining wells. The gel was immersed in TAE buffer and subjected to a voltage difference of 100 volts to separate the fragments. Visualization of the gel was performed under ultraviolet transillumination in a Multiimage Light Cabinet (AlphaInnotech) and the image captured by Alphaimager computer software version 5.5 (AlphaEase™, AlphaInnotech).

A preliminary PCR reaction was performed using 10 type strains of streptococci (Figure 15). These were: *S. sanguinis* NCTC 7863, *S. salivarius* NCTC 8618, *S. mitis* NCTC 12261, *S. anginosus* NCTC 10713, *S. oralis* 11427, *S. mutans* NCTC 10449, *S. gordonii* NCTC 7865, *S. parasanguinis* NCTC 55898, *S. cristatus* CR 311 and *S. intermedius* NCDO 2227.

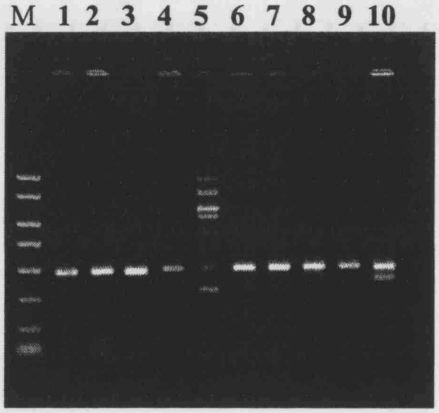


Figure 15: Gel image of *sodA* PCR products of streptococcal type strains

M - Molecular weight marker
1 - *S. sanguinis*
2 - *S. salivarius*
3 - *S. mitis*
4 - *S. anginosus*
5 - *S. oralis*
6 - *S. mutans*
7 - *S. gordonii*
8 - *S. parasanguinis*
9 - *S. intermedius*
10 - *S. cristatus*

Four type strains were selected (two with more than one band and two with one distinct band) and used to optimize the *sodA* PCR protocol. These were *S. salivarius* NCTC 8618, *S. anginosus* NCTC 10713, *S. oralis* NCTC 11427 and *S. cristatus* CR 311. The annealing temperature was raised by 10 degrees to 47°C to minimize nonspecific binding. Four master mixes were made with different concentrations of Taq NH_4 reaction buffer to determine the best PCR product band and to minimize nonspecific binding. The concentrations used were 1, 1.5, 2 and 2.5 × reaction buffer (Figure 16).

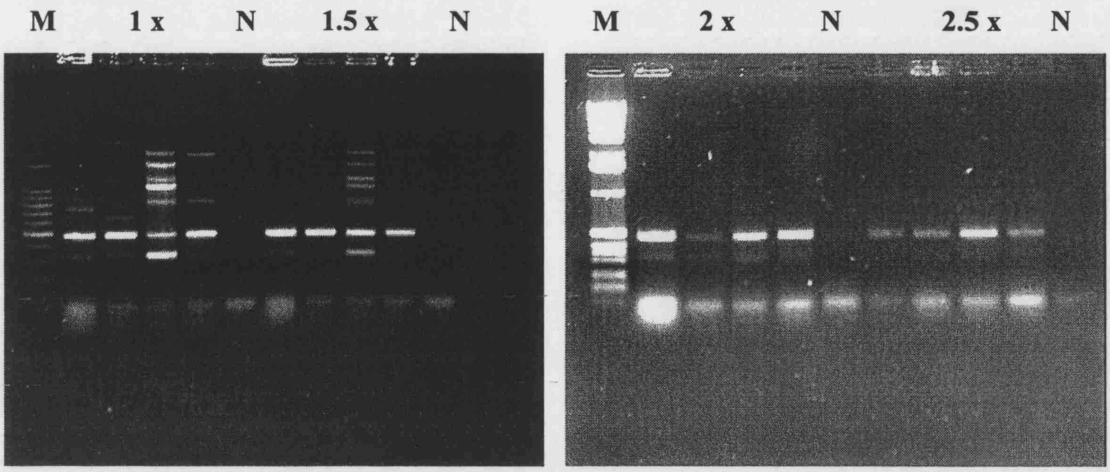


Figure 16: Gel image of *sodA* PCR products of streptococci type strains showing different band strengths resulting from use of 1, 1.5, 2, 2.5 × buffer
(N – negative control, M – molecular weight marker)

***SodA* Gene PCR**

The PCR master mix (50 µl) contained: 0.6 µM of each primer (D1 and D2), 0.4 M dNTPs (dATP, dCTP, dGTP, dTTP; Promega), 2.5 mM $MgCl_2$, 2 × reaction buffer and 1 U Taq Polymerase (Bioline). PCR amplification was performed in a thermal cycler (Primus) according to the following conditions: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 47°C for 90 seconds, 72°C for 90 seconds, followed by an extension period of 72°C for 10 minutes.

***SodA* Gene Sequencing**

The PCR tubes containing 50 µl of PCR product were purified using the Qiagen purification kit (Qiagen) and sequenced using D1 primer (Oswel Company, Eurogentec, Romsey, UK). Resulting electrophoretograms were analyzed in the same manner as described earlier using Chromas version 1.45. Stringent criteria were used and only sequences with 300 or more bases, tall peaks, low background noise and a minimal number of unidentified bases (N's) were submitted to the BLAST analysis database (Altschul et al 1990; Altschul et al 1997). The matches with the highest alignment scores were accepted as final species identification (Appendix 8).

Phylogenetic Trees

The gene sequences were aligned with ClustalX version 1.81 (Thompson et al 1997). The resulting sequence alignment was edited using BioEdit version 5.0.9 (Hall 1999). Phylogenetic analysis was performed with the PHYLIP suite of programs (Phylogeny Inference Package, version 3.5c, Department of Genetics, University of Washington, Seattle, WA, USA). The

relative distances were calculated using the DNADIST program and a phylogenetic tree was constructed with NEIGHBOR. The resulting tree was displayed with TREEVIEW (Page 1996).

Biochemical Methods: Carbohydrate Fermentation and Enzyme Hydrolysis

Each isolate was identified using a combination of carbohydrate fermentation tests, hydrolysis of aesculin and arginine and pre-formed glycosidic enzyme activities (Whiley et al 1990a; Beighton et al 1991a; Beighton et al 1991b).

Presumptive streptococci were subcultured into 4 ml of sterile Todd-Hewitt broth (Oxoid) and incubated at 37°C for 48 hours. The broths were checked for purity by Gram-staining and inoculated onto CBA and incubated anaerobically for 24 hours at 37°C. Any contaminated cultures were subcultured until pure.

Carbohydrate Fermentation Tests

The production of acid from amygdalin, arbutin, inulin, lactose, mannitol, melibiose, N-acetylglucosamine, raffinose, sorbitol and glucose (Sigma) was determined using a sterile microtitre tray format (Nalge, Nunc, Denmark). The carbohydrates were prepared using 2.4 g of thioglycollate broth (Difco) without dextrose or indicator, 1.6 g of purple broth base (Difco), 100 ml of distilled water and 1.0 g carbohydrate. The fermentation of glucose was used as an indicator of viability of the inoculum and tests were repeated on isolates with a negative result. One hundred and twenty-five microlitres of each carbohydrate were aliquoted in a well of the same column on each microtitre tray. Forty-five microlitres of each broth culture were inoculated into the wells of a single row with the last row in each tray containing carbohydrate only acting as a negative control (Figure 17). The trays were incubated for 24 hours anaerobically at 37°C. A positive result was indicated by a change in colour from purple to yellow, signifying acid production and a final pH less than 5.2 (Figure 18).

Hydrolysis of Aesculin and Arginine

Aesculin and arginine (Table 32) were placed in 125 µl volumes in the next two columns following the carbohydrates in the microtitre tray. Forty five microlitres of broth were added to each well in the same manner as for the carbohydrates (Figure 17). Aesculin hydrolysis was indicated by a change in colour to black. Arginine hydrolysis was noted by a change in colour to orange following the addition of 25 µl of Nessler's reagent (BDH) to the well (Figure 18).

Table 32: Aesculin and Arginine Preparation

Aesculin	Arginine
1.0 g Tryptone 0.5 g Yeast extract 1.0 g Sodium acetate 0.05 g Ferric ammonium citrate 0.5 g Aesculin 0.1 ml Tween 80 0.5 ml Salt A 0.5 ml Salt B 100 ml distilled water	0.5 g Peptone 0.3 g Yeast extract 0.3 g Glucose 1.0 g Sodium acetate 0.3 g L-arginine 0.1 ml Tween 80 0.5 ml Salt A 0.5 ml Salt B 100 ml distilled water

Salt A	Salt B
0.16 g $CaCl_2$ 0.16 g $MgSO_4$ 400 ml distilled water	0.8 g K_2HPO_4 8.0 g $NaHCO_3$ 1.6 g NaCl 0.8 g KH_2PO_4 400 ml distilled water

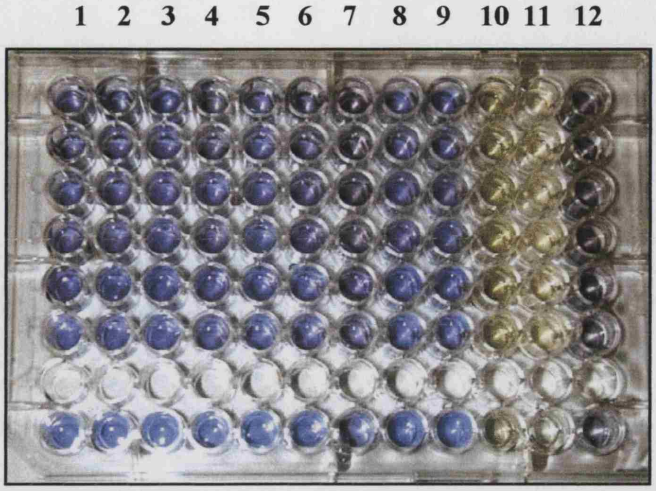


Figure 17: Microtitre tray showing uninoculated carbohydrates (column 1-9), aesculin (column 10), arginine (column 11) and glucose (column 12)

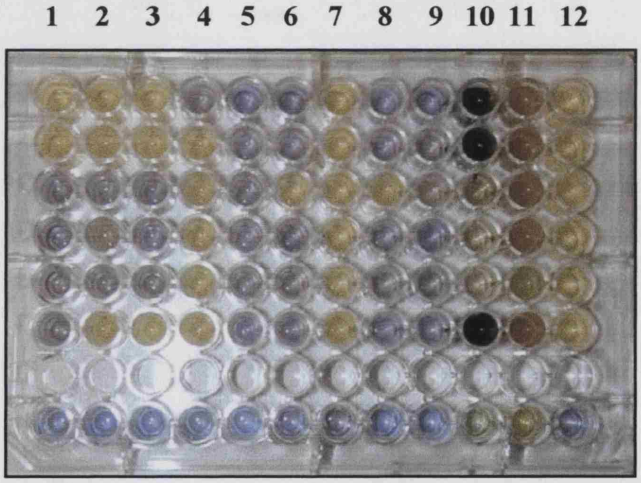


Figure 18: Microtitre tray following inoculation with streptococci broth cultures and anaerobic incubation for 24 hours

Detection of Preformed Enzyme Activity

Bacteria were tested for their ability to hydrolyze a series of 4-methylumbelliferyl (MU-) linked substrates (Sigma) thereby indicating the presence of activity of the following enzymes: β-D-fucosidase, β-N-acetylgalactosaminidase, α-neuraminidase, α-L-fucosidase, β-N-acetylglucosaminidase, α-glucosidase, β-glucosidase, α-galactosidase, α-arabinosidase and β-galactosidase. Each enzyme substrate was prepared from powder by dissolving 1 mg substrate in 0.5 ml dimethyl sulphoxide. This was then added to 9.5 ml of 50 mM N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES buffer) to give a stock concentration of 1 mg/ml. A final working concentration of 100 μg/ml was prepared by adding 1 ml of stock solution to 9 ml of TES buffer.

Each bacterial isolate was subcultured on CBA and incubated anaerobically for 24 hours. Using a sterile swab, the culture was suspended in 1.5 ml of TES buffer to give a turbidity equivalent to MacFarland standard 2. In the same manner as for the carbohydrate fermentation test, 20 µl of enzyme substrate and 45 µl of bacterial suspension were added to the wells in a non-sterile, flat-bottomed microtitre tray (Whiley et al 1990a; Beighton et al 1991a). The last row in each series of plates contained substrate only and was a control. The plates were incubated aerobically for 3 hours at 37°C. Substrate hydrolysis was determined by viewing the trays under an ultraviolet transilluminator in a Multimedia Light Cabinet (AlphaInnotech) using the Alphaimager 1220 version 5.5 computer software program (Figure 19). A bright blue fluorescence of 50 arbitrary units above the control signified that approximately 20 nmoles of substrate had been degraded and was indicative of a positive result. This increase in fluorescence was caused by release of 4-methylumbelliferone (Beighton et al 1991b).

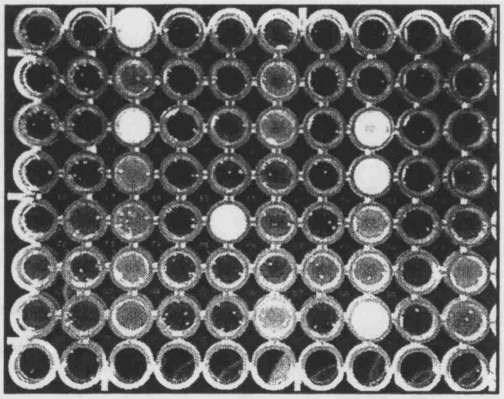


Figure 19: Enzyme hydrolysis viewed using an ultraviolet transilluminator

Regular quality control testing was performed using type strains. These were: *S. sanguinis* NCTC 7863, *S. salivarius* NCTC 8618, *S. mitis* NCTC 12261, *S. anginosus* NCTC 10713, *S. oralis* 11427, *S. sobrinus* NCTC 12279, *S. mutans* NCTC 10449, *S. gordonii* NCTC 7865, *S. parasanguinis* NCTC 55898, *S. cristatus* CR 311, *S. constellatus* NCDO 2226, *S. intermedius* NCDO 2227 and *S. vestibularis* NCTC 12166. The type strains were obtained from the National Collection of Type Cultures (NCTC).

RESULTS

(1) 16S rRNA gene sequencing

Initial identification was by partial sequencing of the 16S rRNA gene. From a total of 90 isolates (representative of 332 cfu's) only 11 were identified to species level. These were *S. sanguinis* (n = 4), *S. parasanguinis* (n = 3), *S. mutans* (n = 2), *S. gordonii* (n = 1) and *S. salivarius* (n = 1). The remaining 78 sequences showed low discrimination between the species with identical scores and e-values when analysed by BLAST (Altschul et al 1990; Altschul et al 1997). The results were used to confirm the genus for further identification and antibiotic sensitivity testing. The phylogenetic tree of streptococcal type strain 16S rRNA gene sequences is shown in Figure 20.

(2) *sodA* gene sequencing

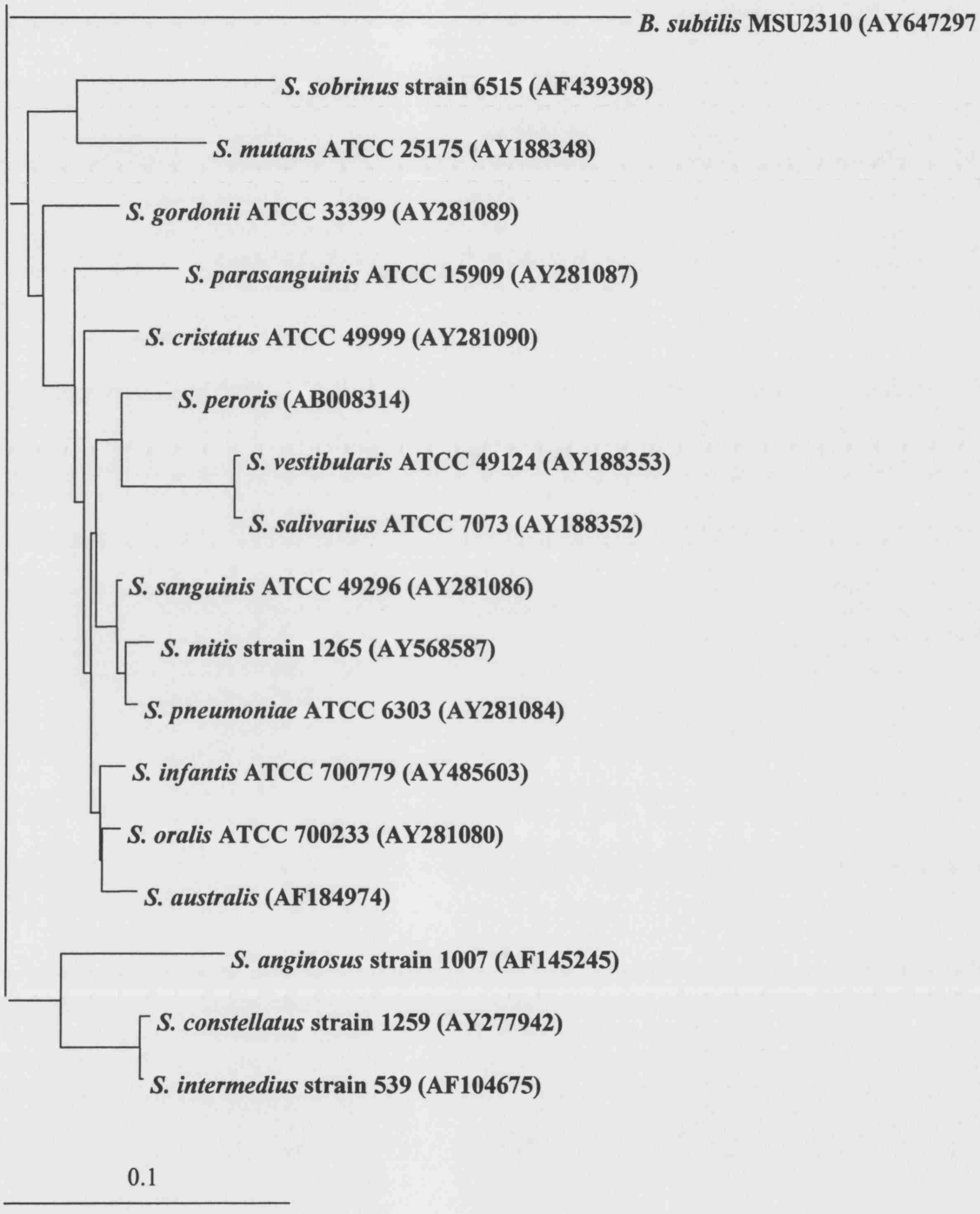
Seventy-one streptococci (representative of 309 cfu's) were speciated by sequencing the *sodA* gene. The results of phylogenetic analysis of type strains and clinical isolates are shown in Figure 21. The clinical strains are presented as HBS (Hawazen Bacteraemia Study) four digit codes (Table 33).

Table 33: Clinical Isolate Code Key

Code	Represents
First digit	Group 1 - Baseline 2 - Post-procedure
Second digit	Procedure group 1 - Rubber dam 2 - Fast drill 3 - Slow drill 4 - Matrix band and wedge 5 - Gingival retraction cord
Third digit	Patient number
Fourth digit	Isolate number

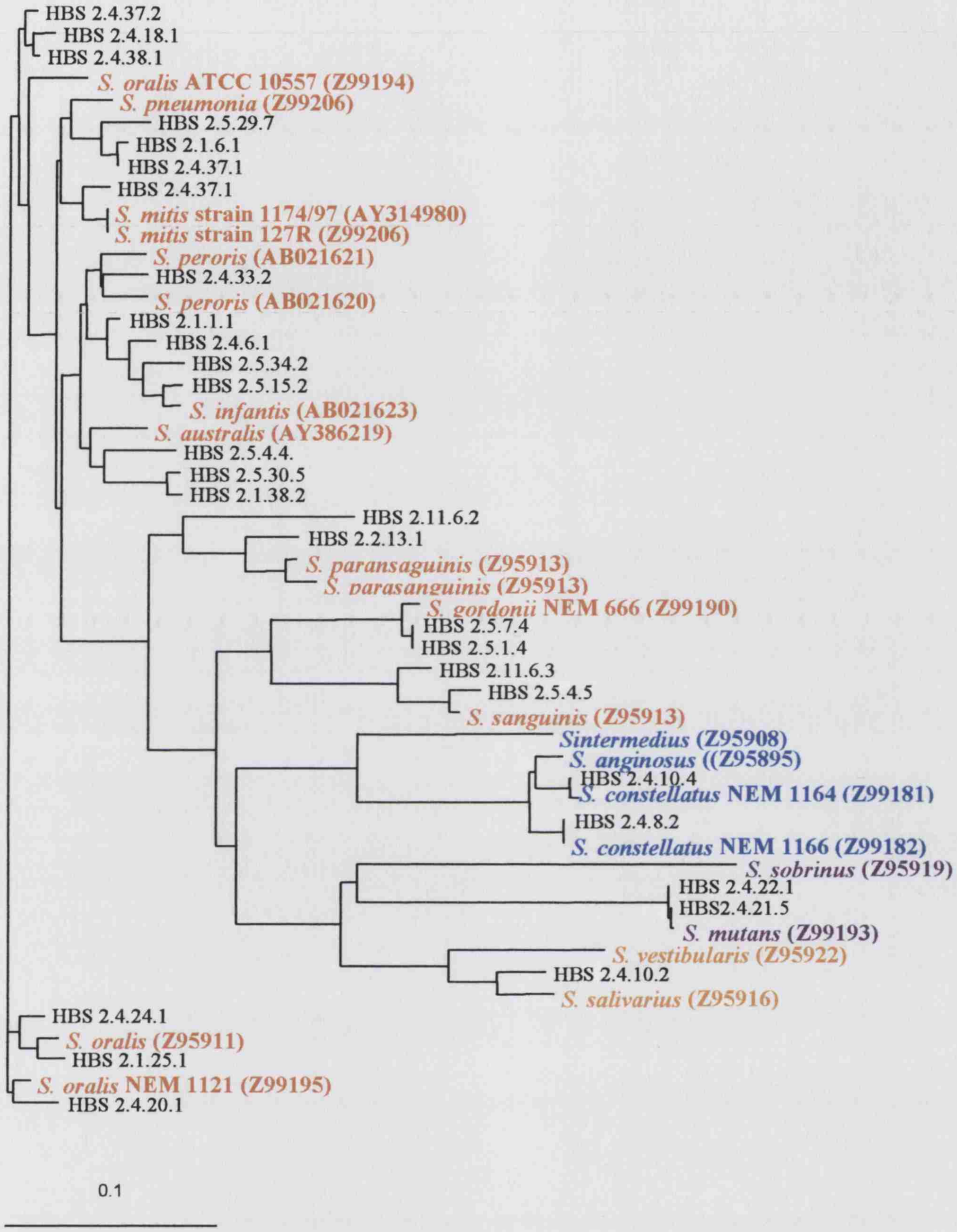
The most commonly isolated species were *S. oralis* and *S. mitis*. The number of cfu's isolated of each streptococcal species is shown in Table 34.

Figure 20: Phylogenetic tree of *Streptococcus* spp. using 16S rRNA gene sequences



Phylogenetic tree based on 16S rRNA gene sequence comparisons over 440 aligned bases of *Streptococcus* spp. GenBank accession numbers of streptococcal type strain sequences from clone libraries are shown in parentheses. The bar represents 10% differences in nucleotide sequences. *Bacillus subtilis* is included as an outgroup.

Figure 21: Phylogenetic tree of *Streptococcus* spp. using *sodA* gene sequences



Phylogenetic tree based on *sodA* gene sequence comparisons over 311 aligned bases of *Streptococcus* spp. Streptococci sequences from clone libraries are colour-coded to distinguish subgroups (mitis group, anginosus group, salivarius group, mutans group) and GenBank accession numbers are shown in parentheses. The bar represents 10% differences in nucleotide sequences.

Table 34: *Streptococcus* spp. Identified by *sodA* Sequencing at Baseline and Following Each Dento-Gingival Manipulative Procedure

Streptococci	Baseline	Post-procedure					Total cfu's
		Rubber dam	Fast drill	Slow drill	Matrix band and wedge	Gingival retraction cord	
S. oralis	0	9	1	1	124	5	140
S. mitis	0	44	0	1	39	3	87
S. gordonii	1	3	0	0	3	11	18
S. sanguinis	0	6	0	0	11	1	18
S. parasanguinis	0	12	1	0	0	1	14
S. pneumoniae	0	1	0	0	4	0	5
S. infantis	1	1	0	0	0	3	5
S. australis	0	4	0	0	0	0	4
S. peroris	0	2	0	0	0	0	2
S. constellatus	0	0	0	0	4	0	4
S. salivarius	0	0	0	0	3	0	3
S. mutans	0	0	0	0	9	0	9
Unidentified *Strep.**	0	8	0	0	9	6	23

*Could not be recovered from frozen culture

(3) Carbohydrate Fermentation and Enzyme Hydrolysis

Seventy-one *Streptococcus* spp. were identified using carbohydrate fermentation and enzyme hydrolysis tests (Whiley et al 1990a; Beighton et al 1991a; Beighton et al 1991b). These were compared with *sodA* gene sequence identification (Table 35). Thirty-nine (55%) of the isolates were identified as the same species following both sequencing of the *sodA* gene and biochemical tests. Eleven isolates, which did not have a published biochemical profile, could not be identified by carbohydrate fermentation and enzyme hydrolysis. Five of these isolates gave an inconclusive profile using carbohydrate fermentation and enzyme hydrolysis. These were identified by *sodA* sequencing as *S. infantis* (n = 3), *S. peroris* (n =1) and *S. pneumoniae* (n =1).

Table 35: Identification of Oral Streptococci: Agreement Between Carbohydrate Fermentation and Enzyme Hydrolysis versus *sodA* Gene Sequencing

Species	Carbohydrate Fermentation and Enzyme Hydrolysis (n)	*sodA* (n)	Agreement (n)
S. oralis	14	24	9
S. mitis	9	10	4
S. oralis/S. mitis	4	-	-
S. gordonii	5	8	5
S. sanguinis	7	5	3
S. parasanguinis	11	6	6
S. pneumoniae	-	4	0
S. peroris	-	2	0
S. infantis	-	3	0
S. australis	-	2	0
S. anginosus	2	0	0
S. constellatuss	2	3	2
S. salivarius	8	2	2
S. vestibularis	1	0	0
S. mutans	2	2	2
S. sobrinus	1	0	
No ID	5	-	-

n = number of isolates

Comparison of *sodA* Gene Sequencing and Carbohydrate Fermentation and Enzyme Hydrolysis

There were a number of differences between the two methods for identification of oral streptococci isolated (Table 36). These are as follows:

Mitis group

Sixty-four (90%) were identified as mitis group using *sodA* sequencing.

(1) Three isolates of *S. oralis* identified by sequencing the *sodA* gene were identified as *S. oralis/S. mitis* by carbohydrate fermentation and enzyme hydrolysis. Four isolates were identified as *S. mitis,* three as *S. salivarius,* two as *S. sanguinis*, two as *S. parasanguinis* and one as *S. anginosus* by biochemical methods.

(2) Three isolates of *S. mitis* identified by sequencing the *sodA* gene were identified as *S. oralis* using biochemical tests. Two isolates of *S. mitis* were identified as *S. salivarius* by biochemical methods and one was identified as *S. sobrinus.*

(3) Three isolates of *S. pneumoniae* from *sodA* gene sequencing were identified as *S. oralis* or *S. mitis* using biochemical tests and one isolate had an inconclusive profile.

(4) Newly identified species of the mitis group, without published carbohydrate fermentation and enzyme hydrolysis profiles, were identified as the following:

(i) One isolate of *S. peroris* from *sodA* gene sequencing was identified as *S. vestibularis* using biochemical methods. A second isolate had an inconclusive profile.

(ii) One isolate of *S. australis* from *sodA* sequencing gave a biochemical profile matching that of *S. sanguinis* and the second isolate a profile matching *S. parasanguinis.*

(iii) Three isolates of *S. infantis* had an inconclusive biochemical profile.

(5) The six isolates of *S. parasanguinis* identified by *sodA* gene sequencing had the appropriate biochemical profiles using carbohydrate fermentation and enzyme hydrolysis tests.

(6) Three isolates of *S. sanguinis* identified by *sodA* gene sequencing matched with the biochemical profile for *S. sanguinis*. One other had a profile matching that of *S. parasanguinis* and one further isolate was identified as *S. salivarius* using carbohydrate fermentation and enzyme hydrolysis tests.

(7) Five isolates of *S. gordonii* were the same with both *sodA* gene sequencing and carbohydrate fermentation and enzyme hydrolysis tests. Three isolates of *S. gordonii* had biochemical profiles of *S. parasanguinis*, *S. sanguinis*, and *S. oralis*, respectively.

Mutans group

The two isolates of *S. mutans* were similarly identified using both *sodA* gene sequencing and carbohydrate fermentation and enzyme hydrolysis.

Salivarius group

Two isolates identified as *S. salivarius* by *sodA* sequencing were similarly identified using biochemical methods.

Anginosus group

Two isolates were identified as *S. constellatus* by both *sodA* sequencing and biochemical methods. One isolate of *S. constellatus* had a biochemical profile matching *S. anginosus*.

Table 36: Identification of Oral Streptococci: Species Differences Between *sodA* Gene Sequencing and Carbohydrate Fermentation and Enzyme Hydrolysis

sodA gene sequence ID	No. misidentified (%)	Misidentified by carbohydrate fermentation and enzyme hydrolysis as species (no.)									
		S.mitis	*S.oralis*	*S.oralis /S mitis*	*S.sanguinis*	*S.parasanguinis*	*S.vestibularis*	*S.salivarius*	*S.anginosus*	*S.sobrinus*	Inconclusive profile
S. oralis	15 (63)	4	-	3	2	2	-	3	1	-	-
S. mitis	6 (60)	-	3	-	-	-	-	2	-	1	-
S. gordonii	3 (38)	-	1	-	1	1	-	-	-	-	-
S. sanguinis	2 (40)	-	-	-	-	1	-	1	-	-	-
S. parasanguinis	0	-	-	-	-	-	-	-	-	-	-
S. pneumoniae	4 (100)	1	1	1	-	-	-	-	-	-	1
S. peroris	2 (100)	-	-	-	-	-	1	-	-	-	1
S. infantis	3 (100)	-	-	-	-	-	-	-	-	-	3
S. australis	2 (100)	-	-	-	1	1	-	-	-	-	-
S. constellatus	1 (33)	-	-	-	-	-	-	-	1	-	-
S. salivarius	0	-	-	-	-	-	-	-	-	-	-
S. mutans	0	-	-	-	-	-	-	-	-	-	-

DISCUSSION

Oral streptococci are principal members of the indigenous microflora of the oral cavity (McCarthy et al 1965; Babaahmady et al 1997; Wilson et al 2002). In certain circumstances, for example immunosuppression, they can become opportunisitic pathogens (Bochud et al 1994; Marron et al 2000). They are also associated with dental caries (Loesche 1986; Becker et al 2002) and purulent infections of oral and non-oral sites (Kuriyama et al 2000; Corson et al 2001; Woo et al 2004). Oral streptococci have been isolated from blood cultures following a variety of dental procedures (Coulter et al 1990; Roberts et al 2000; Lucas et al 2002b). *S. sanguinis*, *S. oralis*, *S. mitis*, *S. gordonii* and *S. parasanguinis* have been identified as causal organisms in IE (Douglas et al 1993; Kikuchi et al 1994).

Recent taxonomic studies of streptococci have resulted in the recognition of several new clinically important species, for example *S. parasanguinis* (Whiley et al 1990b) and *S. gordonii* (Kilian et al 1989), and the redefinition of older ones, for example *S. mitis* (Kilian et al 1989). Based on 16S rRNA gene sequence analysis, the genus *Streptococcus* has been separated into six major clusters or species groups (Kawamura et al 1995b). At close relatedness, 16S rRNA has been found to have low resolving power and, in extreme cases, two species may share identical 16S rRNA gene sequences (Fox et al 1992). This has been reported for species of the mitis group of streptococci which exhibit greater than 99% 16S rRNA sequence homology (Kawamura et al 1995b). Similar findings have been reported for enterococci (Patel et al 1998) and CNS (Poyart et al 2001). Recently 16S rRNA sequences of some species of streptococci were found to be identical to those of other species. This was especially true for *S. mitis*, *S. pneumoniae* and *S. gordonii* (Bosshard et al 2004; Neeleman et al 2004). In the present study, only 12% of oral streptococci were identified to species level using 16S rRNA partial gene sequencing. The remainder showed low discrimination between the species, with identical scores and e-values when analysed by BLAST (Altschul et al 1990; Altschul et al 1997).

SodA gene sequencing was used to speciate the oral streptococci isolated in this study. It has been reported to clearly differentiate *S. mitis*, *S. oralis* and *S. pneumoniae* compared with 16S rRNA gene sequencing (Poyart et al 1998). Some authors believe there might be too much variation within the same species, possibly due to geographic differences as a result of the high evolutionary rate of the *sodA* gene (Kawamura et al 1999). To verify this, oral streptococci clinical strains from different areas, mainly Japan and the UK, were examined but failed to reveal any obvious geographic variation (Kawamura et al 1999). For this reason, it is believed that *sodA* gene sequencing could be applied globally. In the present study,

definitive species identification of oral streptococci was established by *sodA* gene sequence analysis.

Few workers have compared biochemical methods, namely API ID 32 Strep and *sodA* gene sequencing (Poyart et al 1998). Discrepancies between the *sodA* sequencing and API ID 32 Strep system were observed for species with an API identification percentage of less than 99.9% (Poyart et al 1998). Researchers found that an isolate identified as *S. salivarius* by API was identified as *S. mitis* by *sodA* sequencing (Poyart et al 1998). This was similar to the present study where two isolates of *S. mitis* and three of *S. oralis* were misidentified as *S. salivarius* by carbohydrate fermentation and enzyme hydrolysis. Four isolates of *S. oralis* and three of *S. mitis* were identified as the other by biochemical methods. One isolate identified as *S. anginosus* by biochemical methods in the present study was identified as *S. constellatus* by *sodA* sequencing. Different workers found the opposite, with *S. constellaus* identified by the API system then identified as *S. anginosus* by *sodA* gene sequencing (Poyart et al 1998). They also found that certain strains of *S. constellatus*, *S. gordonii*, *S. mitis* and *S. parasanguinis* identified by *sodA* sequencing could not be identified using the API system. The authors considered *sodA* sequencing to be more reliable than the API ID 32 Strep system.

Phenotypic identification schemes have not kept pace with taxonomic developments in the genus *Streptococcus*. They do not allow for unequivocal identification of certain streptococcal species, in particular those belonging to the anginosus (Ahmet et al 1995; Sultana et al 1998), mutans (Hillman et al 1989) and mitis groups (Kawamura et al 1998b). Inherent problems of phenotypic identification systems include the following. Not all strains within a given species exhibit a common characteristic (Kilian et al 1989; Beighton et al 1991b; Pearce et al 1995; Hohwy & Kilian 1995; Pan & Caufield 2001). The same strain may give different results upon repeated testing (Tardif et al 1989; Rudney & Larson 1994). The available biochemical profiles or databases may not include newly or not yet described species. Finally, the test results rely on individual interpretation and expertise. In a recent study, phenotypic characterization using the API 20 Strep system identified 39% of catalase-negative Gram-positive cocci to species level and 19% to genus level while the remainder (42%) could not be discriminated at any taxonomic level (Bosshard et al 2004).

In the present study, 26 of 53 (49%) mitis group streptococci which have known biochemical profiles were misidentified by carbohydrate fermentation and enzyme hydrolysis. Three newly identified species of the mitis group, *S. infantis*, *S. australis* and *S. peroris*, could not be identified by biochemical methods. This is in agreement with other researchers who

observed less than 79% accuracy within the mitis group using the ID 32 Strep and Streptogram systems (Kawamura et al 1998b). Another group of workers found that certain strains of streptococci assigned to the same species by ribotyping were placed in different species by the API Rapid Strep system (Rudney & Larson 1994). This 'phenotypic shift' was mostly seen in *S. sanguinis* and *S. oralis,* which they believed had greater phenotypic variation compared with other species. DNA-DNA hybridization methods have revealed that many strains identified as *S. mitis*, *S. oralis* and *S. sanguinis* by phenotypic methods had been misidentified (Ezaki et al 1988; Kikuchi et al 1995). This is in agreement with results of the present study where *S. oralis*, *S. mitis* and *S. sanguinis* were the most frequently misidentified species by carbohydrate fermentation and enzyme hydrolysis.

Researchers comparing phenotypic characteristics and DNA-DNA hybridization results of 'viridans' group streptococci found that *S. parasanguinis* was not accurately identified using the API ID 32 Strep system (Kikuchi et al 1995). This was because there is no profile for *S. parasanguinis* in the database. The authors believed that if the biochemical profile was included in the API ID 32 system it would be correctly identified because the profile is well characterized. Their hypothesis seems to be well founded as all six isolates of *S. parasanguinis* were correctly identified by carbohydrate fermentation and enzyme hydrolysis in the study reported here.

The mitis group of streptococci was isolated from the saliva of 100% of children aged 6 to 24 months (Könönen et al 2002) and the subgingival plaque of 100% of children aged 7-8 years (Kamma et al 2000). In the present work, the majority of the streptococci isolated were members of the mitis group. This is in agreement with earlier investigations of bacteraemia following dental procedures (Coulter et al 1990; Roberts et al 1997; Roberts et al 1998a; Lucas & Roberts 2000; Roberts et al 2000; Daly et al 2001). Not all bacteraemia studies include speciation of streptococci. From investigations which did include speciation, *S. sanguinis*, *S. mitis* and *S. oralis* were the most frequently isolated streptococci (Coulter et al 1990; Roberts et al 1997; Roberts et al 1998a; Lucas & Roberts 2000). Many of these researchers used commercial identification systems such as API Strep 20 (Coulter et al 1990; Roberts et al 1998a; Roberts et al 2000) which have been shown to have limited sensitivity compared with molecular techniques, especially in identifying mitis group species (Kikuchi et al 1995; Bosshard et al 2004).

In the study reported here, *S. oralis* was the most frequently isolated species accounting for 45% of the oral streptococci identified to species level. Researchers have shown that *S. oralis* is a significant cause of IE (Douglas et al 1993; Doyuk et al 2002) and a major

pathogen in patients with attenuated host defence mechanisms (Beighton et al 1994; West et al 1998). *S. mitis* and *S. oralis* were the most commonly isolated streptococcal species from saliva of healthy children and adults; 42% and 23% respectively (Tappuni & Challacombe 1993). The difference between the reported isolation of *S. mitis* from saliva (Tappuni & Challacombe 1993) and from blood in the present study (28%) may be due to poor retrieval of *S. mitis* from blood or lysing solution. A recent investigation of lysis filtration sensitivity found that from blood samples seeded with type strains of streptococci, *S. mitis* was recovered from 60% following lysis filtration and 85% from broth culture. In comparison, 100% recovery for *S. oralis*, *S. sanguinis*, *S. intermedius* and *S. mutans* was documented (Lucas et al 2002a). Similarly, researchers investigating bacteraemia using lysis filtration in conjunction with endodontic therapy isolated *S. mitis*, *S. sanguinis* and *S. intermedius* from the root canal of single rooted teeth but only isolated *S. sanguinis* from blood samples taken during endodontic treatment (Debelian et al 1995).

Using checkerboard DNA probe hybridization, *S. oralis*, *S. mitis*, *S. sanguinis* and *S. mutans* were the most frequently identified *Streptococcus* spp. from tooth and tongue surfaces of children aged 19 to 36 months (Tanner et al 2002). In the same study, *S. pneumoniae* was isolated from both tooth and tongue surfaces of half the children. *S. pneumoniae* constituted 1.5% of streptococci isolated from the saliva of 50 healthy infants in a recent investigation (Könönen et al 2002). In the present study, *S. pneumoniae* was isolated from two patients following placement of a matrix band and wedge.

S. intermedius comprised a large proportion of the *Streptococcus* spp. isolated from blood following dental extractions in adults (Heimdahl et al 1990; Rajasuo et al 2004). Although *S. intermedius* was not isolated from subjects in this study, another member of the anginosus group, *S. constellatus,* was isolated from two patients following matrix band and wedge placement.

Among the possible virulence factors in oral streptococci causing IE, most attention has been given to adherence properties (Douglas 1994; Baddour 1994). These include interaction with subendothelial components, plasma proteins, platelets, intracellular polysaccharides and lipoteichoic acid (Douglas 1994). *S. sanguinis*, *S. gordonii* and *S. mitis* bind to laminin via a surface protein. In the case of *S. gordonii* the laminin binding protein is only induced when grown in the presence of laminin (Sommer et al 1992). Streptococcal adherence to fibronectin exposed or deposited at sites of endothelial damage may also be important in the pathogenesis of IE (Baddour 1994). Fibronectin binding proteins have been identified in

S. sanguinis (Lowrance et al 1990), *S. gordonii* (McNab et al 1996) and *S. anginosus* (Willcox et al 1995).

The ability to aggregate platelets has also been associated with the capacity to cause IE (Douglas 1994). *S. sanguinis* has been shown to possess the ability to bind and aggregate platelets (Herzberg et al 1992; Manning et al 1994). Another group of workers found no clear correlation between platelet aggregation and the severity of experimental IE caused by *S. constellatus*, *S. intermedius* and *S. anginosus* (Kitada et al 1997). Recently, streptococcal binding interactions were identified in *S. mitis* (Bensing et al 2001).

Potential virulence factors expressed by *S. oralis* include binding to fibronectin, fibrin and platelets (Douglas 1994). This species alone accounted for 60% of the streptococci isolated following matrix band and wedge placement. *S. sanguinis*, *S. gordonii* and *S. mitis* show many virulence properties that may account for their prominence as causes of IE. These three species collectively accounted for 59%, 34% and 53% of streptococci isolated following placement of rubber dam, matrix band and wedge and gingival retraction cord, respectively.

Summary

Species identification of oral streptococci was established by *sodA* gene sequence analysis. The most frequently isolated species were *S. oralis* (45%) and *S. mitis* (28%).

CHAPTER 6
IDENTIFICATION OF STREPTOCOCCI USING RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

INTRODUCTION

It is becoming increasingly important to find rapid and accurate identification methods for oral streptococci due to the increasing incidence of antibiotic resistance (Carratalá et al 1995) and recognition of species-specific virulence factors (Jenkinson & Lamont 1997). *SodA* partial gene sequencing has been found to be a reliable and practical method for accurate identification of oral streptococci, including the closely related mitis group (Poyart et al 1998; Kawamura et al 1999; Poyart et al 2002). In the previous chapter it was used successfully to identify oral streptococcal clinical isolates.

A limitation of gene sequencing is that the cost and accessibility to the appropriate equipment is restricted to advanced molecular reference laboratories. In addition, paying for an offsite service is often expensive. These problems are compounded if sequencing is to be carried out in a developing country where these facilities are scarce or unavailable. In such cases an alternative less expensive and less complicated technique would be ideal.

One alternative technique is restriction fragment length polymorphism (RFLP) analysis. This technique involves cleavage of PCR-generated gene amplicons by restriction endonucleases. Endonucleases are enzymes produced by various bacteria that cut DNA at specific recognition sequences composed of four to six base pairs. This results in the generation of several fragments, the size of which is determined by the content of recognition sequences within the molecule. Separation of the fragments is achieved by electrophoresis in an agarose gel stained with ethidium bromide. The differences (polymorphism) in the size of the DNA fragments generate specific band patterns on the gel. The advantage of RFLP analysis is that it is a simple technique which can be performed in the majority of laboratories with a PCR machine and gel electrophoresis equipment. In addition, a large number of isolates can be analyzed at the same time with results available within 24 hours.

RFLP of the 16S rRNA gene has been reported as a method of bacterial species identification for oral treponemes (Sato & Kuramitsu 1999), *Capnocytophaga* spp. (Ciantar et al 2001), *Eubacterium* spp. (Sato et al 1998), *Nocardia* spp. (Conville et al 2000), *Bifidobacterium* spp. (Delcenseri et al 2004), *Campylobacter* and *Helicobacter* spp. (Marshall et al 1999). Other genetic targets for RFLP analysis include the 16S-23S rDNA intergenic spacer region (Rachman et al 2004), the heat shock protein (HSP) gene (Steingrube et al 1997), and the

hupB gene encoding a histone-like protein (Prabhakar et al 2004). RFLP of an amplified region of the *sodA* gene for identification of streptococci has been recently developed (Jaffray 2003). The aim of this part of the investigation was to determine the usefulness of *sodA* PCR–RFLP by comparing band patterns of streptococci identified to species level using *sodA* gene sequencing with established type strain band patterns (Jaffray 2003).

MATERIALS AND METHODS

Streptococcal clinical isolates identified to species level using *sodA* gene sequencing were subjected to *sodA* PCR–RFLP. In addition, eight type strains were included. These were: *S. oralis* NCTC 11427, *S. mitis* NCTC 12261, *S. pneumoniae* NCTC 7465, *S. gordonii* NCTC 7865, *S. salivarius* NCTC 8618, *S. mutans* NCTC 10449, *S. sanguinis* NCTC 7863 and *S. parasanguinis* NCTC 55898. These were compared to established type strain band patterns (Jaffray 2003) to assess intra-examiner reproducibility. *SodA* PCR–RFLP was performed twice for *S. mitis* NCTC 12261 to confirm reproducibility of the technique.

SodA Gene PCR

Single bacterial colonies were suspended in a PCR master mix (50 µl) containing the following: 0.6 µM of each primer (D1 and D2), 0.4 M dNTPs (dATP, dCTP, dGTP, dTTP; Promega), 2.5 mM $MgCl_2$, 2 × reaction buffer and 1 U Taq Polymerase (Bioline) as described in chapter 5. PCR amplification was performed in a thermal cycler (Primus) using the following conditions: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 47°C for 90 seconds, 72°C for 90 seconds followed by an extension period of 72°C for 10 minutes.

PCR products of approximately 500 bp were assessed using gel electrophoresis in a 1% agarose gel (Amresco) made with TAE buffer (Eppendorf) containing ethidium bromide at a concentration of 0.5 µg/ml. The end well was loaded with a molecular weight marker (50-2000 bp; Amresco). Five microlitres of PCR product and 1 µl of loading buffer (Sigma) were loaded in the remaining wells. The gel was immersed in TAE buffer and subjected to a voltage difference of 100 volts to separate the fragments. Visualization of the gel was performed under ultraviolet transillumination in a Multiimage Light Cabinet (AlphaInnotech) and the image captured by CCD camera and manipulated using Alphaimager software version 5.5 (AlphaEase™, AlphaInnotech).

Double Restriction Digest and RFLP

PCR products were subjected to a restriction digest using restriction endonucleases *Alu* I and *Bgl* II (New England Biolabs, Hitchin, Hertfordshire, UK) (Jaffray 2003). The PCR–RFLP reaction mixture (total volume of 25 µl) contained 10 µl PCR product, 11.5 µl water, 2.5 µl 1 × buffer and 1 µl of each restriction enzyme. The reaction mixture was pulse spun and incubated overnight at 37°C. The resulting digests were mixed with 1 µl loading buffer (Sigma) and loaded into separate wells of 3% superfine resolution agarose (Amresco) gel made with Tris-borate EDTA buffer (TBE; 0.045 M Tris-borate, 0.01 M EDTA) containing 0.5 µg/ml ethidium bromide solution. A molecular weight marker (Hyperladder V; Bioline) with fragments ranging from 25 to 500 bp was loaded into each of the end wells of the gel. The products were subjected to electrophoresis at 60 volts for 3 hours and visualized as described earlier. Band patterns for each *Streptococcus* spp. were compared with type strain patterns (Figure 22) (Jaffray 2003).

RESULTS

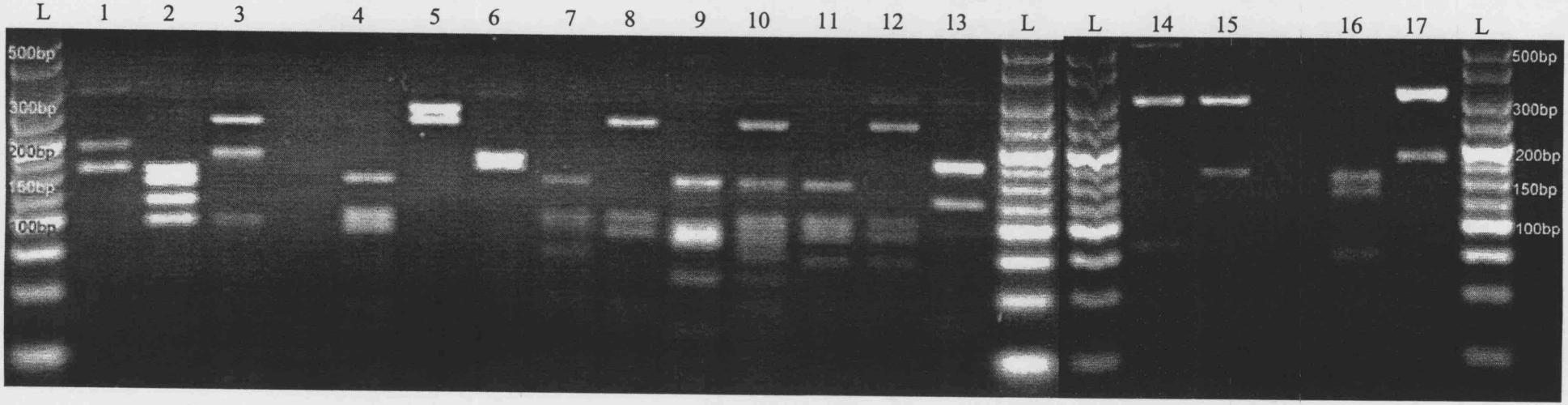
***SodA* PCR-RFLP**

Fifty-six clinical isolates of streptococci were subjected to *sodA* gene PCR–RFLP analysis. Figure 23 shows an example of the appearance of *sodA* RFLP on the gel. The band migration distances were converted to molecular weight sizes using the molecular weight marker as a guide and represented graphically to enable comparison (Figures 24–29).

Type Strain Comparisons

S. sanguinis, *S. parasanguinis*, *S. gordonii*, *S. mutans*, *S. pneumoniae*, *S. salivarius*, *S. mitis* and *S. oralis* type strain RFLP band patterns matched those of established profiles (Jaffray, 2003).

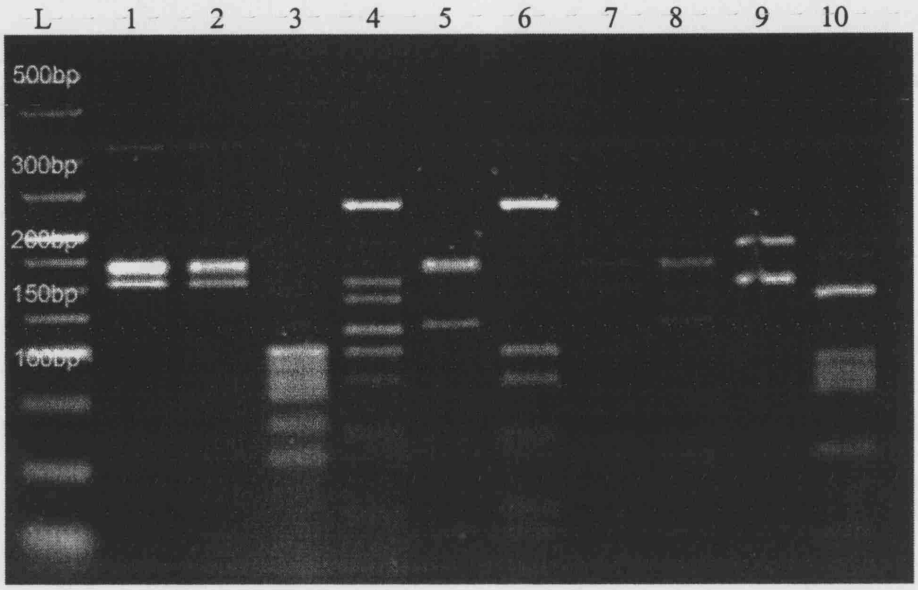
Figure 22: Streptococci type train RFLP profiles (Jaffray 2003)



L - Hyperladder V molecular weight marker

1 - *S. anginosus*
2 - *S. constellatus*
3 - *S. intermedius*
4 - *S. australis*
5 - *S. cristatus*
6 - *S. gordonii*
7 - *S. infantis*
8 - *S. mitis*
9 - *S. oralis*
10 - *S. parasanguinis*
11 - *S. peroris*
12 - *S. pneumoniae*
13 - *S. sanguinis*
14 - *S. mutans*
15 - *S. sobrinus*
16 - *S. salivarius*
17 - *S. vestibularis*

Figure 23: Gel image of *sodA* PCR–RFLP of clinical isolates



L - Hyperladder V molecular weight marker
Clinical isolates identified using *sodA* sequencing as:

1 - *S. gordonii*
2 - *S. gordonii*
3 - *S. mitis*
4 - *S. oralis*
5 - *S. oralis*
6 - *S. mitis*
7 - *S. oralis*
8 - *S. sanguinis*
9 - *S. oralis*
10 - *S. oralis*

Comparison of Type Strain and Clinical Isolate Band Patterns

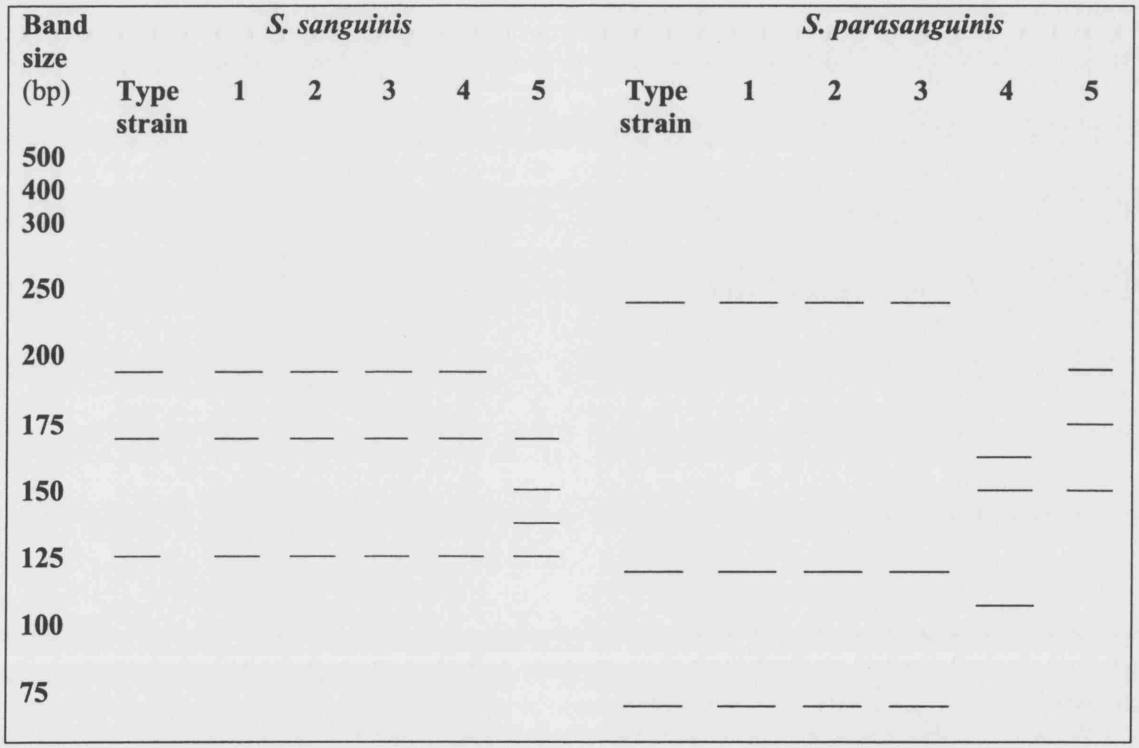
S. sanguinis

Four out of five clinical isolate RFLP band patterns matched with that of the type strain (Figure 24).

S. parasanguinis

Three out of five clinical isolate RFLP band patterns matched with the type strain band pattern (Figure 24).

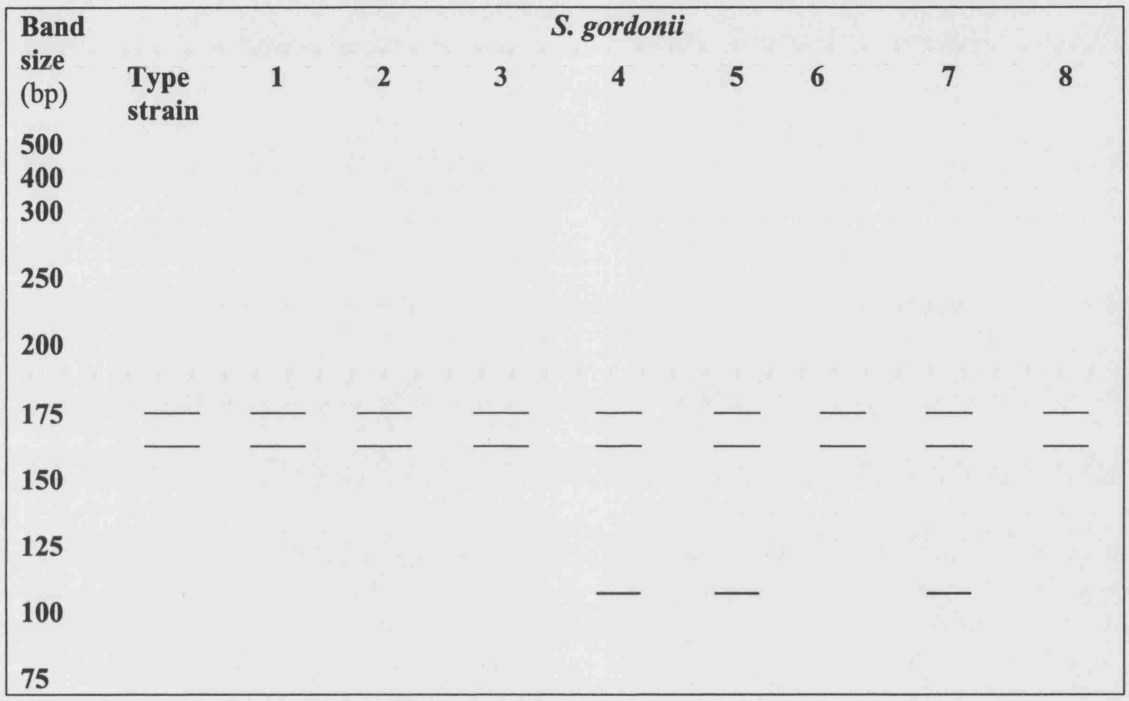
Figure 24: RFLP band patterns for type strains and clinical isolates of *S. sanguinis* and *S. parasanguinis*



S. gordonii

Five out of eight clinical isolate RFLP band patterns exactly matched the type strain band pattern. The remaining three isolates had similar patterns to the type strain profile, but with an extra band at the 110 bp level (Figure 25).

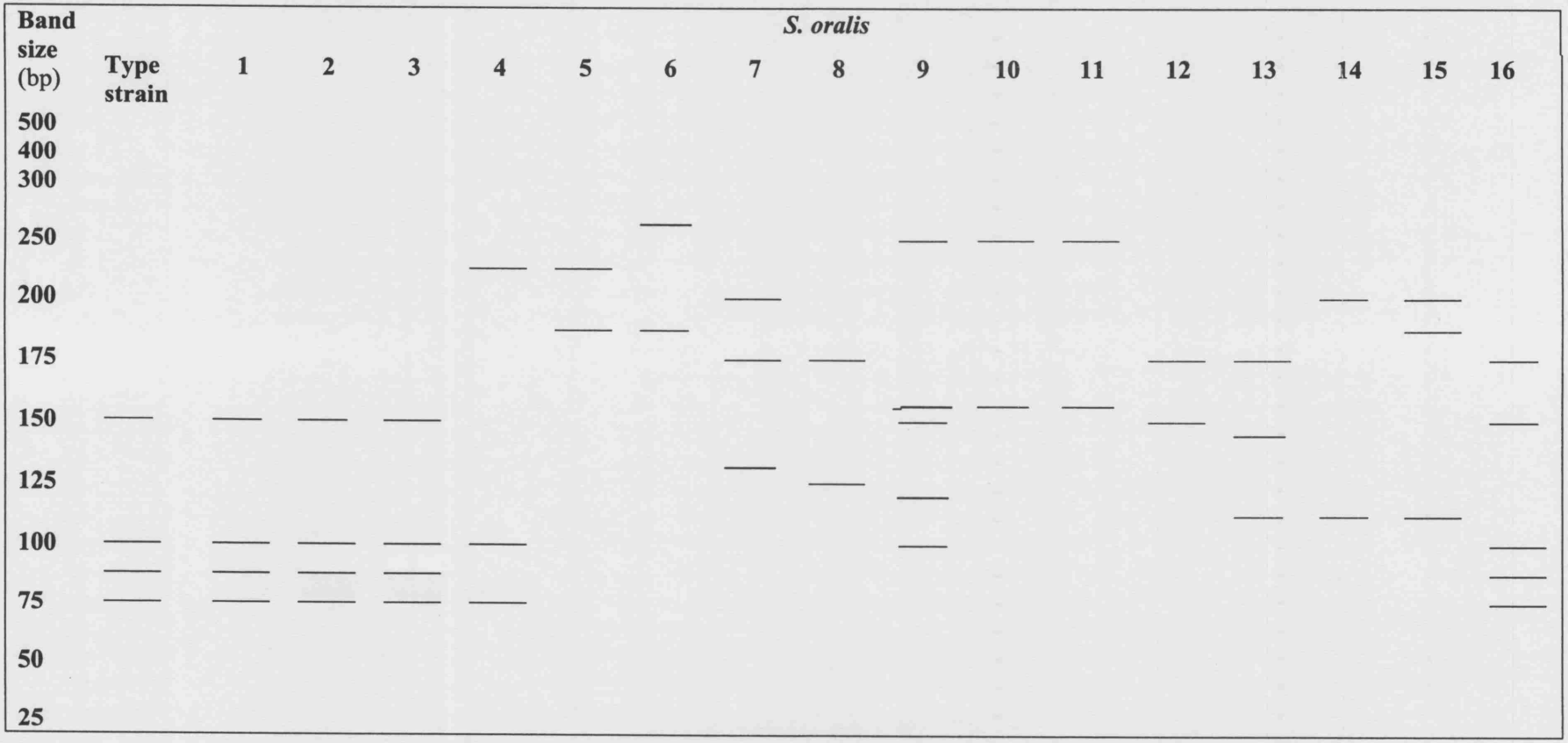
Figure 25: RFLP band patterns for the type strain and clinical isolates of *S. gordonii*



S. oralis

Three of the 16 clinical isolate RFLP band patterns exactly matched that of the type strain. Two isolates (numbers 10 and 11) had the same band pattern. The remainder of the clinical isolates had different RFLP patterns (Figure 26).

Figure 26: RFLP band patterns for type strain and clinical isolates of *S. oralis*



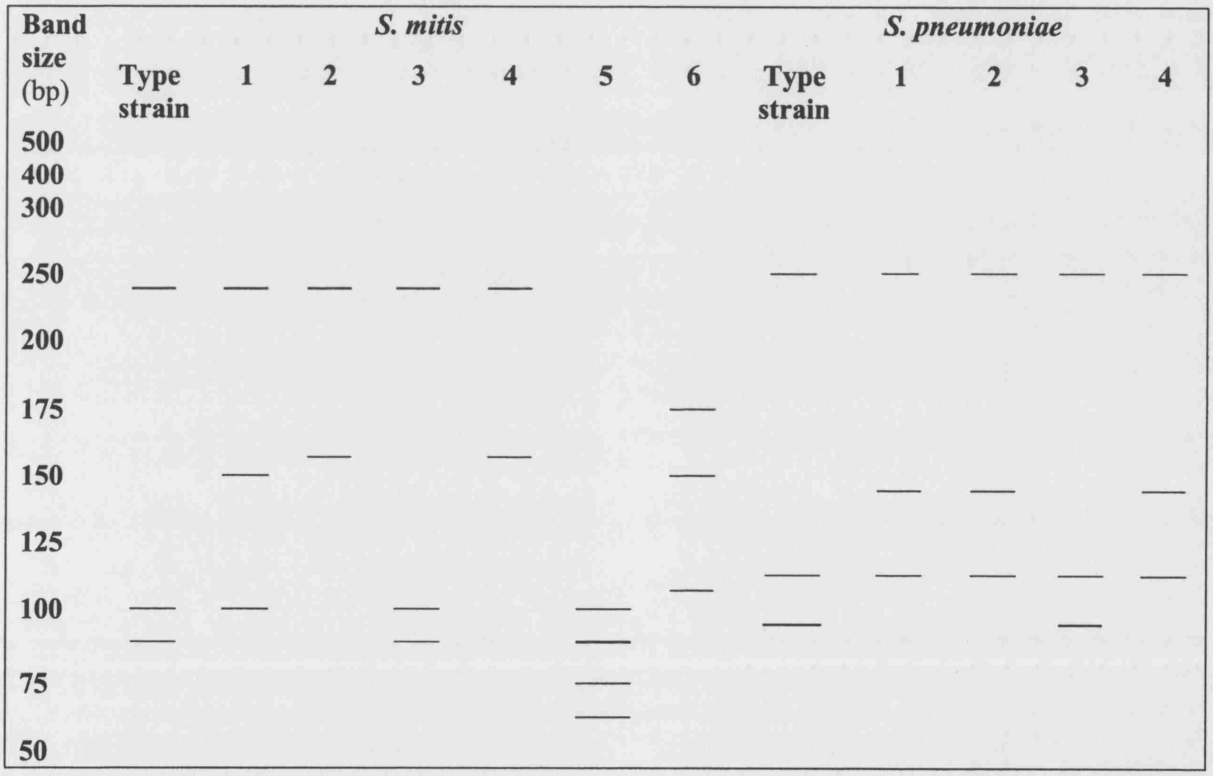
S. mitis

One of the six clinical isolate RFLP band patterns matched that of the type strain. Two isolate band patterns matched each other (numbers 2 and 4) (Figure 27).

S. pneumoniae

One out of four of the clinical isolate band patterns matched that of the type strain. The remaining three clinical isolate band patterns matched each other (Figure 27).

Figure 27: RFLP band patterns for type strains and clinical isolates of *S. mitis* and *S. pneumoniae*



S. peroris

Neither of the two clinical isolate band patterns matched the type strain pattern or each other (Figure 28).

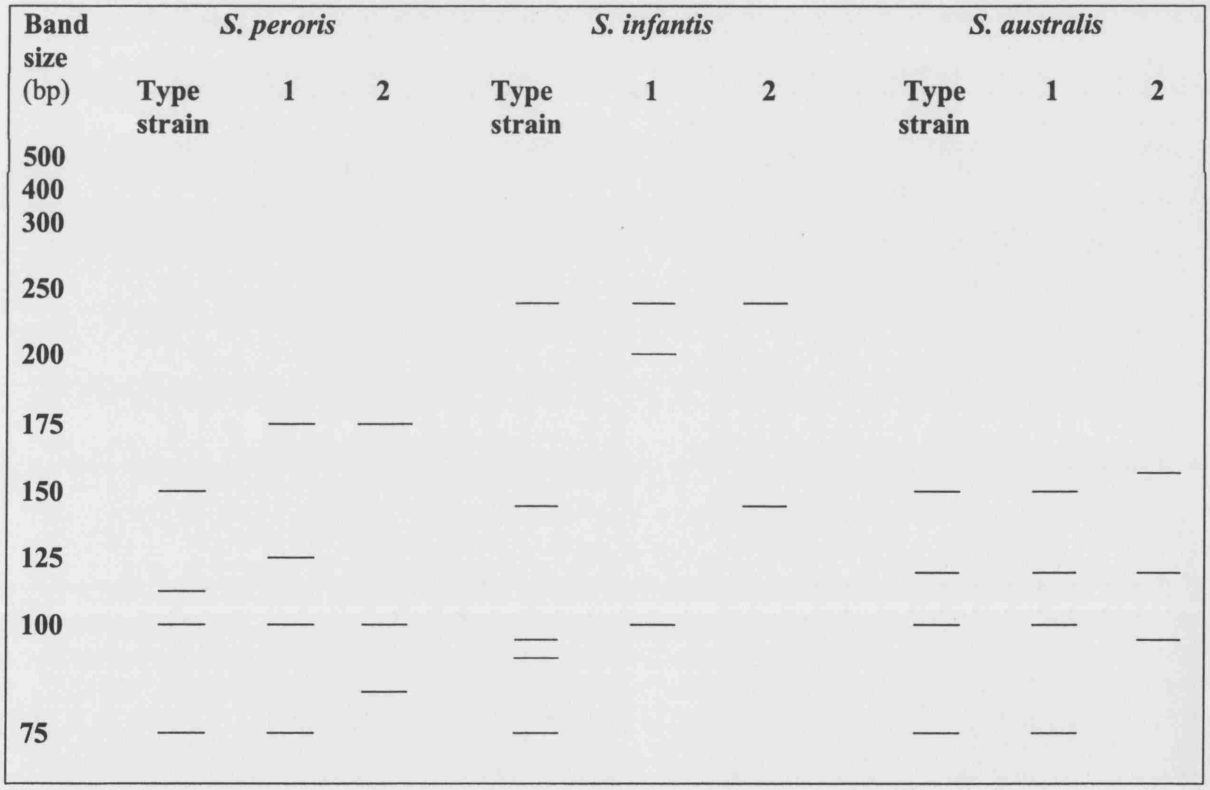
S. infantis

Neither of the two clinical isolate band patterns matched the type strain pattern or each other (Figure 28).

S. australis

One out of two clinical isolates RFLP band patterns matched that of the type strain (Figure 28).

Figure 28: RFLP band pattern for type strains and clinical isolates of *S. peroris*, *S. infantis* and *S. australis*



S. mutans

Both clinical isolate band patterns matched that of the type strain (Figure 29).

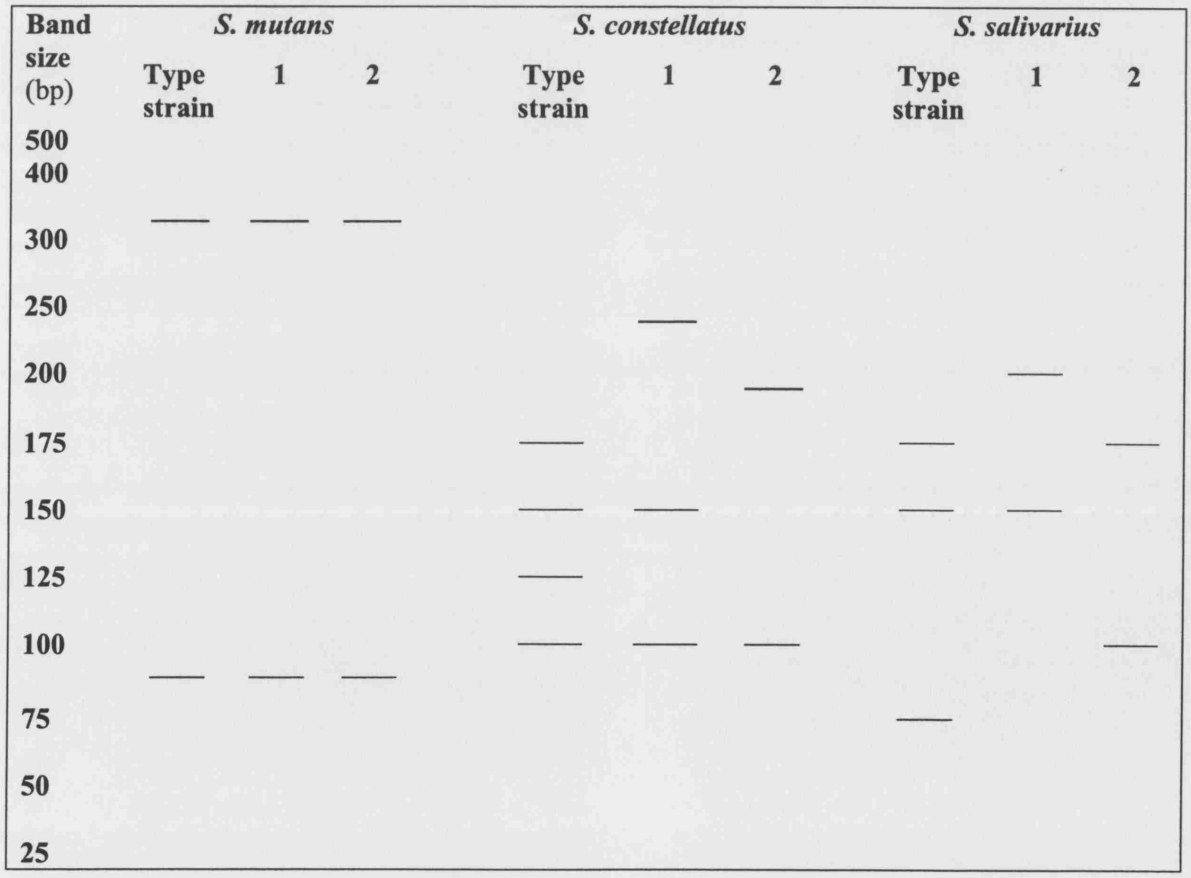
S. constellatus

Neither of the two clinical isolate band patterns matched the type strain pattern or each other (Figure 29).

S. salivarius

Neither of the two clinical isolate band patterns matched the type strain pattern or each other (Figure 29).

Figure 29: RFLP band patterns for type strains and clinical isolates of *S. mutans*, *S. constellatus* and *S. salivarius*



Summary of Results

Table 37 shows a summary of RFLP band pattern results for streptococcal clinical isolates.

Table 37: Summary of RFLP Band Pattern Results

	Number of isolates	Number of band patterns that matched type strain	Number of different band patterns
S. sanguinis	5	4 (80%)	2
S. parasanguinis	5	3 (60%)	3
S. gordonii	8	5 (63%)	2
S. oralis	16	3 (19%)	13
S. mitis	6	1 (17%)	4
S. pneumoniae	4	1 (25%)	2
S. peroris	2	0	2
S. infantis	2	0	2
S. australis	2	1 (50%)	2
S. mutans	2	2 (100%)	1
S. constellatus	2	0	2
S. salivarius	2	0	2

DISCUSSION

The reliability of partial sequence analysis of the *sodA* gene for identification of *Streptococcus* spp. is well documented (Poyart et al 1998; Kawamura et al 1999; Poyart et al 2002). The main aim of this section was to evaluate the potential for using the *sodA* gene as a target for PCR–RFLP analysis for identifying streptococci. This technique would allow rapid and uncomplicated identification of *Streptococcus* spp. without the need for complex and expensive sequencing equipment, which is not available in most developing countries.

PCR–RFLP analysis of type strains for *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. mutans* and *S. salivarius* showed the same band patterns as those described by Jaffray (2003). Closer scrutiny showed great similarity between band patterns of *S. infantis*, *S. peroris* and *S. australis* with differences in the location of one band between the three species. This highlighted the difficulty in interpreting band patterns because of the large number of bands and subtle differences in their location. It was therefore necessary to align each species' RFLP band pattern (Figures 24 to 29). Another difficulty in interpreting RFLP patterns was that very small fragments (less than 25 bp) could not be differentiated well on the gel.

There was variation between the species in the production of the same pattern on the gel by *sodA* PCR–RFLP. For example, 80% of *S. sanguinis* isolates, 60% of *S. parasanguinis* isolates and 63% of *S. gordonii* isolates had RFLP band patterns matching those of their type strain. Both *S. mutans* isolates matched the type strain band pattern. For the 16 *S. oralis* isolates investigated, only three (19%) matched those of the type strain. In addition, there were 13 different band patterns for *S. oralis*. *S. mitis* had four different band patterns resulting from only six isolates with only one of the clinical isolate RFLP band patterns matching that of the type strain. Only one *S. pneumoniae* (n = 4) and one *S. australis* (n = 2) band pattern matched those of the type strain. None of *S. infantis* (n = 2), *S. peroris* (n = 2), *S. salivarius* (n = 2) and *S. constellatus* (n = 2) band patterns matched those of their type strains.

This apparent discrepancy between the species may be explained by the genetic heterogeneity found in certain mitis group species. *S. oralis* has shown the greatest intraspecies divergence between *sodA* fragments (Poyart et al 1998) which suggests that *S. oralis*, as described, is genomically heterogeneous. The close relationship between *S. oralis* and *S. mitis* and *S. pneumoniae* has been shown by approximately 60% overall DNA homology between *S. oralis* and *S. pneumoniae* (Kilpper-Balz et al 1985) and a high level of 16S rRNA gene sequence homology shared by all three species (Kawamura et al 1995b). The results of this part

of the study and the close genetic relationship of *S. mitis*, *S. infantis*, *S. peroris* and *S. australis* to *S. oralis* suggests that they may also be genetically heterogeneous.

In addition, the *sodA* gene has an evolutionary rate that is much faster than that of the 16S rRNA gene which may cause increased genetic variation within the same species (Kawamura et al 1999). As a result of this increased evolutionary rate, genetic mutations occur more frequently. These mutations may be significant enough to cause differences in RFLP patterns but not enough to cause a considerable difference when considering the whole *sodA* gene.

The explanation for the different band patterns of the same species of streptococcus in this study relates to the recognition sites for the restriction endonucleases. RFLP of the *sodA* gene involves cleavage of PCR-generated fragments by restriction endonucleases at specific recognition sequences. Any variation at these sites would affect the restriction point of the enzyme and ultimately the size of the resultant fragments. This leads to endonucleases fragmenting the gene in different areas, dictated by differences in nucleotide bases, producing fragments of different sizes and different band patterns on the gel. On the other hand, *sodA* sequence analysis (Chapter 5) is a base-to-base comparison of the submitted sequence and those in the database. This allows a higher resolution comparison of part of the gene to two or three sites of four to six bp in length which occurs in RFLP analysis.

An interesting finding was that the band pattern for the type strain for *S. gordonii* NCTC 7865 and five of the eight *S. gordonii* clinical isolates showed only two bands at 175 and 160 bp. The size of the *sodA* gene which is amplified should be approximately 480 bp and so the sum of all fragments should be equal to or near 480 bp. *In silico* analysis was performed using the sequence of the *sodA* gene for *S. gordonii,* and endonuclease cleavage points were identified. Three fragments resulted with two fragments nearing 160 bp and one fragment of 175 bp. Therefore, the single band seen at the 160 point actually represents two fragments. As a result of their close size they could not be differentiated on the gel. This is a potential problem for all RFLP analysis.

A possible alternative for RFLP identification of streptococci would be the use of whole genome techniques such amplified fragment length polymorphism (AFLP) analysis (Savelkoul et al 1999). AFLP utilizes a small amount of purified genomic DNA which is digested with two restriction enzymes and subjected to PCR amplification, revealing a highly informative pattern of 40-200 bp. These would provide a greater area of comparison of genetic sequences which is less influenced by minor genetic variation (Neeleman et al 2004). This technique has recently

been shown to be useful in differentiating closely related bacterial species (Chemlal et al 2001; Neeleman et al 2004).

Summary

SodA PCR–RFLP could not produce species-specific band patterns to enable accurate identification of oral streptococci. Although the results for *S. sanguinis*, *S. mutans*, *S. gordonii* and *S. parasanguinis* were promising, *S. oralis*, *S. mitis* and the remainder of the streptococci showed little consistency in their RFLP pattern. This may in part be due to the genetic heterogeneity of this group of microorganisms and the high evolutionary rate of the *sodA* gene.

CHAPTER 7
IDENTIFICATION OF COAGULASE-NEGATIVE STAPHYLOCOCCI TO SPECIES LEVEL

INTRODUCTION

Coagulase-negative staphylococci (CNS) have emerged as important pathogens in hospital acquired infections and IE. CNS are particularly associated with foreign bodies such as prosthetic valves, cerebrospinal fluid shunts, orthopaedic prostheses and indwelling intravenous catheters (Jarvis & Martone 1992; Kloos & Bannerman 1994).

Several commercial methods for identification of staphylococci are available. These include the API (BioMerieux) and Staph-Zym (Rosco, Tastrup, Denmark) identification systems (Ieven et al 1995). The overall accuracy of commercial identification systems is considered to be low for CNS, ranging from 50% to 70% (Perl et al 1994). Several genotypic methods based on analysis of PCR products derived from selected DNA targets have been developed for species-level identification, including determination of 16S rRNA sequences (Zakrzewska-Czerwinska et al 1992). Closely related species may have identical 16S rRNA sequences or divergent 16S rRNA sequences may exist within a single organism (Stackebrandt & Goebel 1994). Use of alternative target sequences with well-conserved DNA sequences within a given species but with sufficient sequence variation to allow for species-specific identification would avoid this problem (Goh et al 1996). Examples of DNA sequences that have been used for CNS identification are the *hsp60* gene (Goh et al 1996), *tuf* gene (Martineau et al 2001) and the *sodA* gene (Poyart et al 2001). Sequencing of the *sodA* gene has been found to be a valuable approach for genotypic identification of both streptococci (Poyart et al 1998) and enterococci (Poyart et al 2000) in addition to staphylococci.

MATERIALS AND METHODS

Sequencing of the *sodA* Gene

Single bacterial colonies were suspended in 50 µl of PCR master mix containing 0.6 µM of each primer, D1 and D2 (Table 38), 0.4 M dNTPs (dATP, dCTP, dGTP, dTTP; Promega), 2.5 mM $MgCl_2$, 1 × reaction buffer and 1 U Taq Polymerase (Bioline). A negative control using sterile, nuclease-free water was prepared for each reaction. PCR amplification was performed in a thermal cycler (Primus) according to the following conditions: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 37°C for 90 seconds, 72°C for 90 seconds, followed by an extension period of 72°C for 10 minutes.

Table 38: Primers used for *sodA* PCR Amplification and Sequencing

Primer	Sequence
D1	5'-CCITAYICITAYGAYGCIYTIGARCC
D2	5- ARRTARTAIGCRTGYTCCCAIACRTC

R=A+G, Y=C+T, I=Inosine

PCR products (approximately 500 bp) were visualized using gel electrophoresis in a 1% agarose gel (Amresco) made with TAE buffer (Eppendorf) containing ethidium bromide at a concentration of 0.5 μg/ml. The end well was loaded with a molecular weight marker (50-2000 bp; Amresco). Five microlitres of PCR product and 1 μl of loading buffer (Sigma) were loaded in the remaining wells. The gel was immersed in TAE buffer and subjected to a voltage difference of 100 volts to separate the fragments. Visualization of the gel was performed under ultraviolet transillumination in a Multiimage Light Cabinet (AlphaInnotech) and the image captured by CCD camera and manipulated using Alphaimager computer software version 5.5 (AlphaEase™, AlphaInnotech).

The PCR mixture was purified using the Qiagen purification kit (Qiagen) and sequenced using D1 primer (Oswel Company, Eurogentec, Romsey, UK). The resulting electrophoretograms were analyzed using the computer software program Chromas version 1.45. Stringent criteria were used and only sequences with 300 or more bases, tall peaks, low background noise and a minimal number of unidentified bases (N's) were submitted to the BLAST analysis database (Altschul et al 1990; Altschul et al 1997). The matches with the highest alignment scores were accepted as final species identification (Appendix 9).

Phylogenetic Trees

The gene sequences were aligned with ClustalX version 1.81 (Thompson et al 1997). The resulting sequence alignment was edited using BioEdit version 5.0.9 (Hall 1999). Phylogenetic analysis was performed with the PHYLIP suite of programs (Phylogeny Inference Package, version 3.5c). The relative distances were calculated using the DNADIST program and a phylogenetic tree was constructed with NEIGHBOR. The resulting tree was displayed with TREEVIEW (Page 1996).

Biochemical Methods

API ID 32 Staph System

Presumptive staphylococci and micrococci were further identified using the API ID 32 Staph system (Biomerieux). This system consists of plastic strips made of 32 cupules of which 26 contain dehydrated biochemical media for colourimetric tests (Figure 30). The tests include acid production from glucose, mannose, fructose, maltose, lactose, trehalose, mannitol, raffinose, sucrose, N-acetylglucosamine, turonase, ribose, arabinose and cellobiose; decarboxylation of arginine and ornithine; production of urease, β-glucoronidase, β-galactosidase, acetoin, alkaline phosphatase, arginine arylamidase and pyrrolidonyl-arylamidase; hydrolysis of aesculin; reduction of nitrate; and susceptibility to novobiocin.

The bacterial suspension was prepared from overnight cultures on CBA by inoculating the bacteria into 3 ml of sterile distilled water to a MacFarland standard of 0.5. The suspension was distributed into the wells of the strip by delivery of 55 µl per well with a pipette. The strips were incubated at 37°C for 24 hours in an aerobic chamber in a sealed container with a small volume of water to prevent dehydration. One drop of reagent (VP A, VP B, NIT 1, NIT 2, FB reagents; Biomerieux) was added for the nonspontaneous tests (nitrate reduction, production of acetoin, galactosidase, arginine arylamidase, alkaline phosphatase and pyrroldonyl arylamidase) as advised by the manufacturer. Strain profiles were read as colour changes 5-10 minutes after addition of the reagents and recorded on the score sheets provided. The tests on the result sheet are divided into groups of three with each assigned a value of 1, 2 or 4 when positive and the values are added together within each group. The resulting nine digit numerical profile was manually entered into the computer using APILAB ID 32 software. This software gives a probability of identification in a range of 10 to 100%.

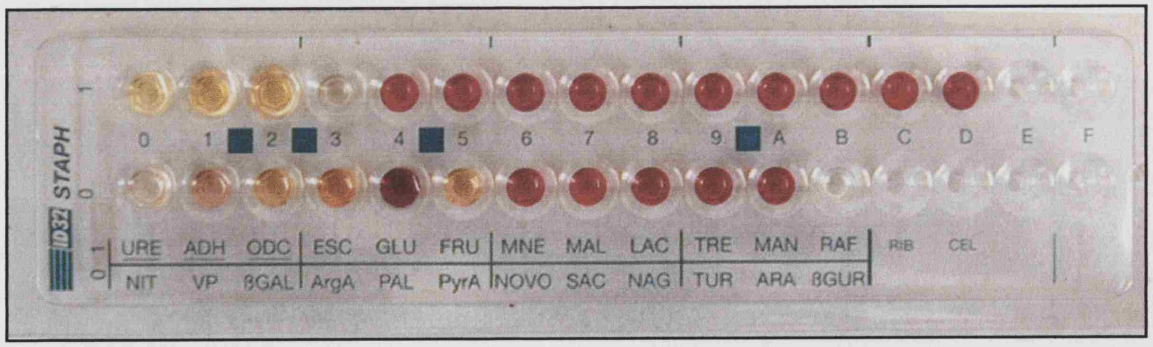


Figure 30: API ID 32 Staph tray following 24 hour incubation

RESULTS

One hundred and eight presumptive staphylococci were isolated. All of these were identified using 16S rRNA and *sodA* gene sequencing. Forty-six staphylococci were also identified using the API ID 32 Staph system.

(1) 16S rRNA partial gene sequencing

All 108 isolates of *Staphylococcus* spp. (representative of 143 cfu's) were sequenced using universal primers 27f and 1492r. The 16S rRNA gene sequences were analysed using the BLAST 2.2.1 analysis database (Altschul et al 1990; Altschul et al 1997). Some species had the same or very similar scores and e-values, particularly for *Staph. hominis* which could not be differentiated from *Staph. xylosus*. In addition, *Staph. epidermidis* could not be differentiated from *Staph. caprae*, *Staph. arlettae*, *Staph. capitis* and *Staph. cohnii*. The results of phylogenetic analysis of type strains and clinical isolates are shown in Figure 31.

(2) *sodA* partial gene sequencing

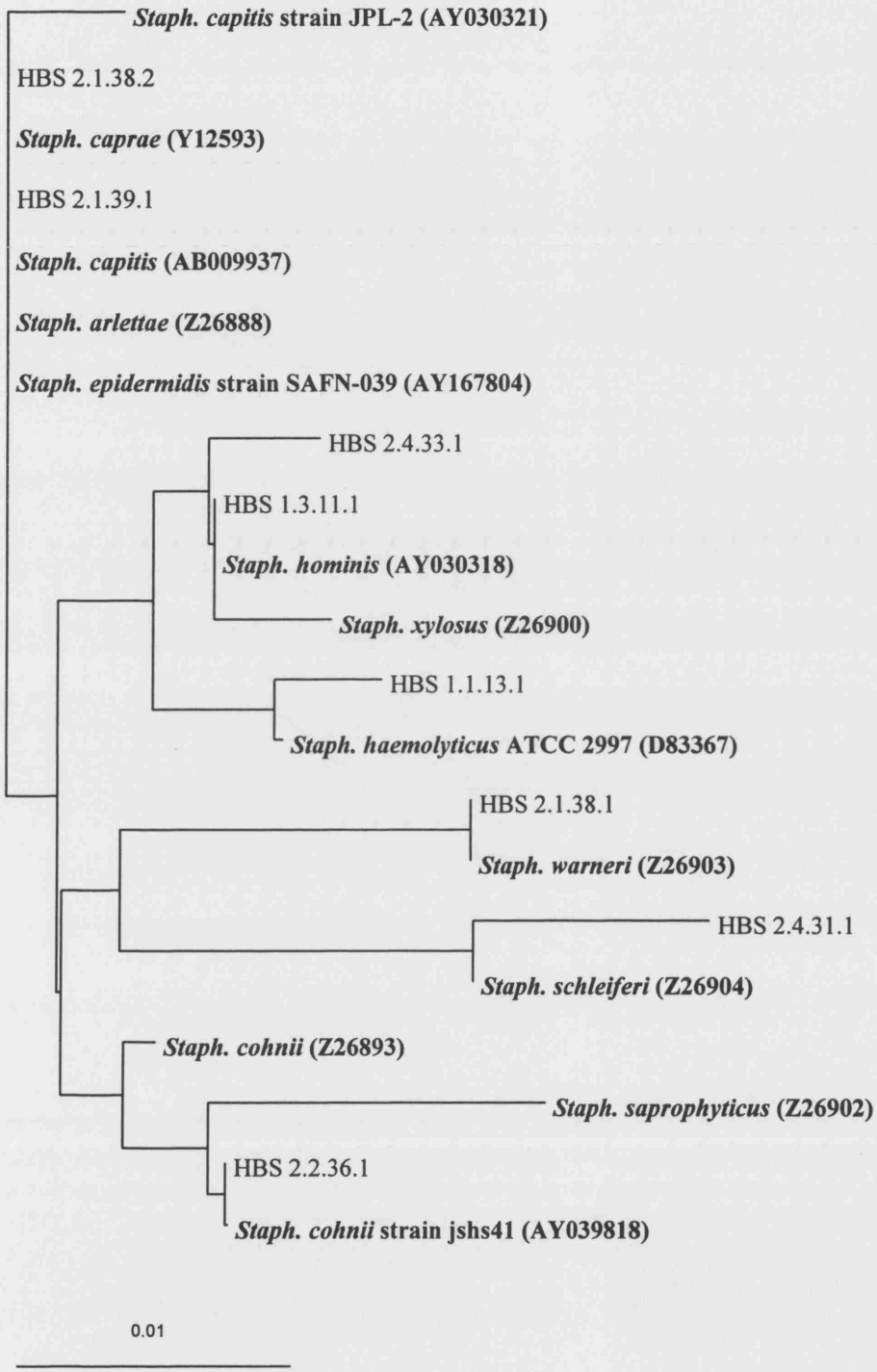
The *sodA* gene was sequenced for all 108 isolates of *Staphylococcus* spp. (representative of 143 cfu's) and the results of phylogenetic analysis of type strains and clinical isolates are shown in Figure 32. The clinical strains are presented as a four digit code, HBS (Hawazen Bacteraemia Study) (Table 39).

Table 39: Clinical Isolate Code Key

Code	Represents
First digit	Group 1 - Baseline 2 - Post-procedure
Second digit	Procedure group 1 - Rubber dam 2 - Fast drill 3 - Slow drill 4 - Matrix band and wedge 5 - Gingival retraction cord
Third digit	Patient number
Fourth digit	Isolate number

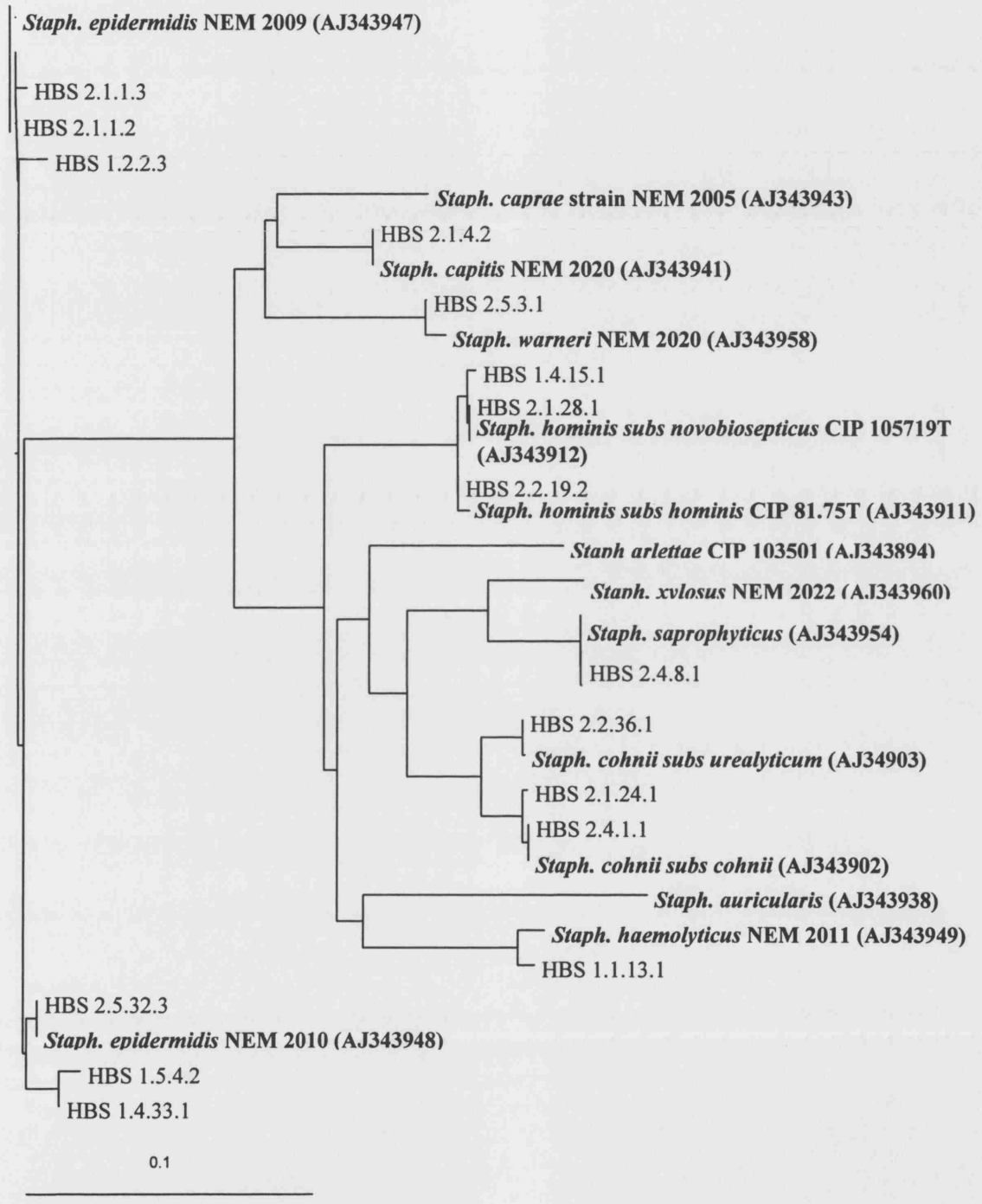
The most commonly isolated species were *Staph. epidermidis* and *Staph. hominis*. The number of cfu's of each CNS species is shown in Table 40.

Figure 31: Phylogenetic tree of *Staphylococcus* spp. using 16S rRNA gene sequences



Phylogenetic tree based on 16S rRNA gene sequence comparisons over 241 aligned bases of *Staphylococcus* spp. Distances were calculated by the neighbour-joining method. Staphylococci sequences from clone libraries are in bold type and GenBank accession numbers are shown in parentheses. The bar represents 1% difference in nucleotide sequences.

Figure 32: Phylogenetic tree of *Staphylococcus* spp. using *sodA* gene sequences



Phylogenetic tree based on *sodA* gene sequence comparisons over 290 aligned bases of *Staphylococcus* spp. Distances were calculated by the neighbour-joining method. *Staphylococcus* spp. sequences from clone libraries are in bold type and GenBank accession numbers are shown in parentheses. The bar represents 10% differences in nucleotide sequences.

Table 40: *Staphylococcus* spp. Isolated at Baseline and Following Each Dento-Gingival Manipulative Procedure

Staphylococci	Baseline	Post-procedure					Total cfu's
		Rubber dam	Fast drill	Slow drill	Matrix band and wedge	Gingival retraction cord	
Staphylococci	34	30	11	5	33	30	**143**
Staph. epidermidis	12	16	2	1	8	12	51
Staph. hominis	17	10	5	4	18	15	69
Staph. warneri	4	2	3	-	4	2	15
Staph. capitis	-	1		-	-	1	2
Staph. cohnii	-	1	1	-	1	-	3
Staph. haemolyticus	1	-	-	-	-	-	1
Staph. saprophyticus	-	-	-	-	1	-	1
Staph. schleiferi	-	-	-	-	1	-	1

(3) Biochemical identification

API ID 32 Staph System

Sixty-two presumptive *Staphylococcus* spp. were identified using the Staph ID 32 API system (Biomerieux). The identification for staphylococci using API was compared with identities based on 16S rRNA and *sodA* gene sequencing. Forty-five (73%) were identified as the same species by all three methods. Using 16S rRNA gene sequencing, only *Staph. warneri* and *Staph. haemolyticus* could be clearly differentiated. All of the staphylococci were speciated by *sodA* gene sequencing and these results were compared with API identification (Table 41). Thirty-four of 46 CNS (74%) showed the same identification by both API and *sodA* gene sequencing.

The identity of 16 isolates using the API system was found to be different from *sodA* gene sequencing. These were:

(1) Three isolates of *Staph. hominis* were identified as *Staph. epidermidis* and one isolate as *Staph. warneri* using *sodA* gene sequencing

(2) Five isolates of *Staph. epidermidis* were identified as *Staph. hominis* by *sodA* gene sequencing

(3) One isolate of *Staph. haemolyticus* was identified as *Staph. warneri* and the other as *Staph. hominis* by *sodA* gene sequencing

(4) One isolate each of *Kocuria varians* and *Kocuria rosea* were identified as *Staph. hominis* by *sodA* gene sequencing

Although *Micrococcus* spp. and *Rothia* spp. are not members of the staphylococcal group, the same biochemical method was used for their identification. Hence the results are presented here. Seven *Micrococcus* spp. and five *Rothia* spp. were identified with differences in identification noted for the following:

(1) One isolate each of *M. lylae* and *M. nishinomiyaenis* were identified as *M. luteus* by 16S rRNA gene sequencing

(2) One isolate of *Rothia mucilaginosa* was identified as *Rothia dentocariosa* by 16S rRNA gene sequencing

(Table 42)

Table 41: Species Identification of *Staphylococcus* spp. and *Micrococcus* spp. by API versus *sodA* Gene Sequencing

	API No. of strains	**16S rRNA** No. of strains	***sodA*** No. of strains	**Agreement** (API and molecular method)
Staph. epidermidis	25	Not differentiated	23	20 (80%)
Staph. hominis	13	Not differentiated	15	8 (62%)
Staph. warneri	2	4	4	2 (50%)
Staph. haemolyticus	3	1	1	1 (33%)
Staph. capitis	1	Not differentiated	1	1 (100%)
Staph. saprophyticus	1	Not differentiated	1	1 (100%)
Staph. cohnii	1	Not differentiated	1	1 (100%)
M. luteus	7	7	NA	7 (100%)
M. lylae	1	0	NA	0
M. nishinomiyaenis	1	0	NA	0
Rothia mucilaginosa	5	4	NA	4 (80%)
Kocuria varians	1	0	NA	0
Kocuria rosea	1	0	NA	0

NA – not applicable – *sodA* sequencing not performed

Table 42: Species Misidentified by API ID 32 Staph

Sequence ID	No. misidentified (%)	Misidentified by API ID 32 Staph as species (no.)						
		Staph. epidermidis	*Staph. hominis*	*Staph. haemolyticus*	*Micrococcus nishinomiyaenis*	*Micrococcus lylae*	*Kocuria* spp.	*Rothia mucilaginosa*
Staph. epidermidis*	3 (13)	-	3	-	-	-	-	-
Staph. hominis*	8 (53)	5	-	1	-	-	2	-
Staph. warneri*	2 (50)	-	1	1	-	-	-	-
***M. luteus*[R]**	2 (29)	-	-	-	1	1	-	-
***R. dentocariosa*[R]**	1 (100)	-	-	-	-	-	-	1

* - *sodA* gene sequence identification
R – 16S rRNA gene sequence identification

DISCUSSION

CNS have been regarded as skin commensals and dismissed as culture contaminants. With the increasing use of prosthetic heart valves and intravascular catheters they are emerging as serious pathogens (Huebner & Goldmann 1999) causing up to 60% of prosthetic valve endocarditis (Rubinstein & Carbon 1998). Although CNS have been a less frequent cause of native valve endocarditis, the incidence is now increasing (Etienne & Eykyn 1990; Miele et al 2001).

Identification of CNS is difficult due to the similarity of biochemical traits between species (Kawamura et al 1998a). In addition, many clinical isolates show intermediate traits (Kawamura et al 1998a). Although commercial identification kits are used by many laboratories, they are not sufficiently accurate for identification of all CNS species (Perl et al 1994; Ieven et al 1995; Renneberg et al 1995). Researchers in Denmark found that both the API ID 32 Staph system and the Staph-Zym system reasonably identified 82% of CNS isolated from blood (Renneberg et al 1995). In the present study, 74% of CNS isolates had the same identities using both the API system and *sodA* gene sequencing. This is in agreement with other researchers who found 74% of CNS isolated from human clinical specimens were correctly identified by conventional tests compared with microplate DNA-DNA hybridization (Kawamura et al 1998a). Researchers comparing phenotypic and genomic methods of identification for CNS found that only 52% of clinical isolates could be identified by the API ID 32 Staph system while 83% were identified by rRNA gene restriction site polymorphism analysis (Chesneau et al 1992).

Sequencing of the 16S rRNA gene region is widely used. Some researchers have encountered problems with closely related species because 16S rRNA is not sufficiently sensitive. These include *Lactobacillus lactis* (Collins et al 1989), *Streptococcus parasanguinis* (Whiley et al 1990b), *Mycobacterium intracellulare* (Boddinghaus et al 1990) and *Bacillus* spp. (Fox et al 1992). In the present study sequencing of the 16S rRNA gene resulted in a lack of differentiation between certain CNS species. This lack of differentiation was apparent as identical alignment scores and e-values. This occurred between *Staph. hominis* and *Staph. xylosus* and between *Staph. epidermidis* and *Staph. capitis*, *Staph. arlettae*, *Staph. cohnii* and *Staph. caprae*. The phylogenetic tree constructed using 16S rRNA gene sequence data shows the lack of discrimination between these species. This has been reported by other workers (Poyart et al 2001; Bosshard et al 2004).

To enable definite species identification, the *sodA* gene was selected as a target for sequencing. This method has been found to be a valuable approach in the genotypic identification of many

closely related species, including CNS (Poyart et al 2001). Sequence analysis and the phylogenetic tree constructed (Figure 32), revealed that staphylococcal *sodA* genes exhibit a higher divergence than the corresponding 16S rRNA sequences. The phylogenetic tree showed a differentiation between the disputed species by 16S rRNA sequencing (*Staph. hominis* and *Staph. xylosus* and *Staph. epidermidis* and *Staph. capitis*, *Staph. arlettae*, *Staph. cohnii* and *Staph. caprae*). Ten percent sequence variation was seen with *sodA* gene analysis compared to only 1% from analysis of 16S rRNA gene sequences. It also enabled the distinction between subspecies of *Staph. hominis* (*Staph. hominis* subsp. *hominis* and *Staph hominis* subsp. *novobiosepticus*) and *Staph cohnii* (*Staph. cohnii* subsp. *cohnii* and *Staph. cohnii* subsp. *urealyticum*). The results of the present study confirm that the *sodA* gene constitutes a more discriminative target than the 16S rRNA gene to differentiate CNS. As a result, CNS species designation by *sodA* partial gene sequencing was considered as final species identification.

Ninety-eight percent of clinically significant blood isolates from patients with suspected bacteraemia in the United States were identified as *Staph. epidermidis*, *Staph. hominis* and *Staph. haemolyticus* (Weinstein et al 1998). These three species accounted for 84% of CNS isolates in this part of the study. The most frequently isolated species was *Staph. hominis* accounting for 48% of all CNS isolates. *Staph. hominis* comprised 6-17% of staphylococci isolated from the subgingival crevice (Rams et al 1990; Murdoch et al 2004). In the above studies, the API system was used to identify CNS to species level. This method has been found to be inaccurate for *Staph. hominis* identification. Fifty-three percent of *Staph. hominis* in the present study were misidentified by the API ID 32 Staph system when compared with *sodA* gene sequencing. Other researchers have made similar observations where 67% (Ieven et al 1995) and 40% (Renneberg et al 1995) of *Staph. hominis* were identified incorrectly by the API system. *Staph. hominis* was also identified with the least accuracy using the Vitek Systems Gram-Positive Identification Card (Bannerman et al 1993) and Rapid and Conventional MicroScan Panels (Weinstein et al 1998). *Staph. hominis* may be more prevalent in clinical samples than believed, resulting from inaccuracies in the currently used identification systems.

In the present study, 36% of CNS isolates were *Staph. epidermidis*. Eighty-seven percent of *Staph. epidermidis* were correctly identified by the API system in this study. This is in agreement with an earlier investigation in which 89% of *Staph. epidermidis* were correctly identified by conventional methods (Kawamura et al 1998a). Although not entirely comparable, *Staph. epidermidis* constituted 67–80% of isolates from subgingival plaque in a recent study (Murdoch et al 2004). *Staph. epidermidis* is the most commonly reported CNS species causing IE (Richardson et al 1984; Hoen et al 2002). In a recent investigation, *Staph. epidermidis* was found to cause 91% of cases of CNS native valve endocarditis (Etienne & Eykyn 1990).

Other researchers reported *Staph. warneri* as 5-11% of the *Staphylococcus* spp. present in the subgingival crevice (Rams et al 1990; Murdoch et al 2004). This is similar to the proportion in this study which is 10%. *Staph. warneri* has also been found to cause significant infections, particularly native valve endocarditis (Dan et al 1984; Caputo et al 1987; Wood et al 1989; Etienne & Eykyn 1990). *Staph. capitis* has been a rare cause of IE, although eight cases of native valve endocarditis due to *Staph. capitis* have recently been reported (Banders & Dariouiche 1992; Lina et al 1992; Latorre et al 1993; Mainardi et al 1993; Sandoe et al 1999). In two of these cases, a history of dental treatment before the development of IE symptoms was recorded (Latorre et al 1993; Mainardi et al 1993). In the work reported here, *Staph. capitis* was isolated from only two patients following rubber dam and gingival retraction cord placement. In most investigations of bacteraemia following dental procedures, the species of CNS have not been specified. Hence comparisons have not been possible.

Not all *Staphylococcus* spp. are equal in their pathogenic potential (Baddour et al 1984). There have been several reports of highly destructive CNS native valve endocarditis caused by *Staph. epidermidis* (Miele et al 2001) and *Staph. lugdunensis* (Kralovic et al 1995; Seenivasan & Yu 2003). A group of researchers compared the virulence of several species of staphylococci and found that *Staph. epidermidis* and *Staph. schleiferi* were more virulent than *Staph. warneri* and *Staph. hominis* (Lambe et al 1990). Similarly another group of workers found *Staph. epidermidis* to be more virulent than *Staph. hominis* in causing IE in a rat. With an inoculum of 10^7 cfu/ml, *Staph. epidermidis* resulted in IE in all the rats while *Staph. hominis* resulted in IE in 40% (Baddour et al 1984). *Staph. epidermidis* was found to be more resistant to phagocytic killing than *Staph. hominis* strains (Lambe et al 1990). The production of slime by *Staph. epidermidis* was suggested as a marker of virulence by another group of workers (Gunn 1989).

Staph. schleiferi, isolated from one patient following matrix band and wedge placement, has been reported to cause IE, osteomyelitis and urinary tract infections (Hernandez et al 2001). The exact pathogenic mechanisms of this species are still unknown but it shares virulence determinants with *Staph. aureus*, namely its ability to express clumping factors and/or produce thermostable DNAase (Vandenesch et al 1994).

Staph. epidermidis has shown considerable virulence in causing IE (Gunn 1989; Lambe et al 1990). This is particularly significant as *Staph. epidermidis* comprised 36% of CNS isolated following dento-gingival manipulative procedures in this study. It also accounts for the majority of IE cases caused by CNS (Richardson et al 1984; Hoen et al 2002).

Summary

Species identification for CNS was established by *sodA* gene sequence analysis. *Staph. hominis* was the most frequently misidentified by the API ID 32 Staph system when compared with *sodA* gene sequencing. The most frequently isolated CNS in the present work were *Staph. hominis* (48%) and *Staph. epidermidis* (36%).

CHAPTER 8
ANTIBIOTIC SENSITIVITY OF ORAL STREPTOCOCCI

INTRODUCTION

Recently, attention has been directed towards antibiotic resistance of oral microorganisms. This may reduce the efficacy of antibiotic prophylaxis for IE and result in treatment failure of orofacial infections.

Penicillin-resistant strains of oral streptococci were first reported in 1963 in the oropharynx of children receiving continuous penicillin prophylaxis for rheumatic fever (Naiman & Barrow 1963). Since then intermediate and high resistance to penicillin has been encountered in several countries (Guiot et al 1994; Carratalá et al 1995; Ioannidou et al 2001). Workers in the United States reported that 56% of viridans streptococci isolated were resistant to Penicillin G (Doern et al 1996) and in Spain 57% were found to be resistant (Carratalá et al 1995). Other investigators in Greece, found that 15% of 200 streptococcal isolates from the oropharynx of healthy children showed high level resistance to penicillin and 29% showed intermediate resistance (Ioannidou et al 2001). Penicillin-resistant streptococci have been isolated from children, with reported resistance of 22% in the Netherlands (Guiot et al 1994) and 13% in Japan (Mogi et al 1997).

Prior administration of penicillin and cephalosporins has been reported to reduce the susceptibility of oral streptococci to ß-lactam antibiotics (Carratalá et al 1995; Kennedy et al 2001). This has been shown to influence the efficacy of these antibiotics in the prophylaxis of streptococcal (Hess et al 1983a; Pujades et al 1990) and staphylococcal IE (Voorn et al 1992). Following the administration of penicillin for prophylaxis in cancer patients, penicillin resistance increased from 0% in 1989 to more than 80% in 1994 (Kremery & Trupl 1995). Patients who receive antibiotic prophylaxis with penicillin have been found to harbour antibiotic-resistant streptococci in the oral cavity (Leviner et al 1987; Fleming et al 1990). A statistically significant increase in the number of subjects harbouring penicillin-resistant oral streptococci following the administration of 2 gm penicillin V and 1 gm 6 hours later on three successive Mondays has been reported. However, the number of penicillin-resistant streptococci as a percentage of the total oral streptococci was small, within the range of 0.0003% to 0.41% (Fleming et al 1990).

Methods of Antibiotic Sensitivity Testing

There are three main methods of antibiotic sensitivity testing:

1. Agar diffusion tests

A defined quantity of antibiotic is allowed to diffuse from a point source, usually in the form of an impregnated filter paper disc, into agar medium that has been inoculated with the test organism. After incubation, a circular zone of growth inhibition appears around the disc. The zone is compared with that obtained with a control isolate and a result recorded of sensitive, intermediate or resistant. There are many technical variables that can affect the zone size and comparability of results. These include solubility, ionic charge and molecular size of the antibiotic agent and the growth rate of the bacteria (Greenwood & Slack 2000). Despite this, agar diffusion remains the most commonly used technique for routine susceptibility testing in the UK (Barker 1999).

2. Broth dilution tests

Serial dilution, usually two-fold, of antibiotic in a suitable fluid medium are inoculated with the test organism. The highest dilution of the antibiotic to prevent the development of visible growth after overnight incubation is the minimum inhibitory concentration (MIC). Disadvantages of this technique are that it is time-consuming and expensive, and only a small number of strains can be tested at a time (Greenwood & Slack 2000).

3. Agar dilution tests

These are similar to broth dilution tests except that the antibiotic dilutions are incorporated in the agar medium in a series of petri dishes. These are spot inoculated with a number of test organisms by means of a semi-automatic inoculating device. This method is time-consuming and labour intensive, requiring media containing the antibiotic solution to be prepared immediately before use.

E test

The Epsilometer test (E test) has been developed for direct quantification of antimicrobial susceptibility of microorganisms. It uses the principles of both broth dilution and agar diffusion. The test consists of thin, non-porous strips 5 mm wide and 60 mm long. One side of the strip carries the MIC reading scale in μg/ml. An exponential gradient of antibiotic at concentrations equivalent to 0.016–256 μg/ml is immobilized on the other side of the strip. When the E test strip is placed on an inoculated agar surface there is immediate transfer of the preformed antibiotic gradient into the agar matrix and the exponential gradient of antibiotic is

formed directly underneath the strip. The E test method has been validated using the agar dilution as control for antimicrobial susceptibility of periodontopathic microorganisms (Nachnani et al 1992) and streptococci isolated from blood (Rosser et al 1999).

MATERIALS AND METHODS

The antibiotic sensitivity of streptococci was determined using E test strips (AB Biodisk, Solna, Sweden). Eleven antibiotics which are included in the British Society for Antimicrobial Chemotherapy Endocarditis Working Party guidelines (Simmons 1993) and the endocarditis prophylaxis guidelines for the American Heart Association (Dajani et al 1997) were used. These were penicillin, amoxicillin, erythromycin, azithromycin, clarithromycin, clindamycin, gentamicin, cephalexin, teicoplanin and vancomycin. Cephazolin was replaced with cephalothin as no E test strips are available for this antibiotic.

Sixty-three oral streptococci were tested for their susceptibility to the above antibiotics. The streptococci tested were *S. oralis* (n = 24), *S. mitis* (n = 8), *S. gordonii* (n = 7), *S. sanguinis* (n = 5), *S. parasanguinis* (n = 4), *S. pneumoniae* (n = 4), *S. infantis* (n = 3), *S. peroris* (n = 2), *S. australis* (n = 2), *S. constellatus* (n = 2), *S. salivarius* (n = 1) and *S. mutans* (n =1).

Type Strains

The type strain included in each batch was *S. oralis* NCTC 11427. Antibiotic sensitivity was performed for the following streptococcal type strains: *S. mitis* NCTC 12261, *S. gordonii* NCTC 7865, *S. salivarius* NCTC 8618, *S. sobrinus* NCTC 12279, *S. mutans* NCTC 10449, *S. sanguinis* NCTC 7863, *S. parasanguinis* NCTC 55898, *S. anginosus* NCTC 10713 and *S. pneumoniae* NCTC 7465.

Determination of Antibiotic Sensitivity using the E test

Preparation of Inoculum for the E test

The inoculum was prepared using direct colony suspension. Each streptococcal isolate was incubated for 24 hours on CBA, Gram-stained and tested for catalase production prior to inoculum preparation. The direct colony suspension method was used as recommended by NCCLS guidelines (National Committee for Clinical Laboratory Standards 2003). Several well isolated colonies were inoculated into Mueller Hinton broth, adjusted to a MacFarland standard of 0.5 and used within 15 minutes of preparation.

E test Method

Mueller Hinton Agar (MHA; Oxoid) supplemented with 5% defibrinated horse blood in a 150 mm plate at a depth of 4.0 ± 0.5 mm was used for inoculation of bacteria. The inoculum was streaked onto the entire agar surface using a sterile cotton swab three times, rotating the plate approximately 90 degrees each time to distribute the inoculum evenly. The plates were allowed to dry for approximately 30 minutes before the E test strips were applied. Four, five or six E test strips were carefully positioned in a radial fashion on each agar plate using sterile forceps.

The plates were incubated for 20-24 hours in 5% CO_2 atmosphere at 35°C. E tests were read using good lighting and a magnifying lens. The MIC value for each antibiotic was recorded as the lowest point where the edge of the elliptical zone of inhibition intersected the MIC scale on the strip (Figure 33). For the glycopeptide antibiotics (vancomycin and teicoplanin) a slim inhibition ellipse results due to the large molecules remaining in the vicinity of the strip, requiring careful scrutinization of endpoints for hazes or microcolonies to verify complete inhibition.

Reproducibility

Antibiotic susceptibility testing to the above antibiotics were repeated on seven occasions for the reference strain of *S. oralis* NCTC 11427 and *Staph. aureus* NCTC 6571.

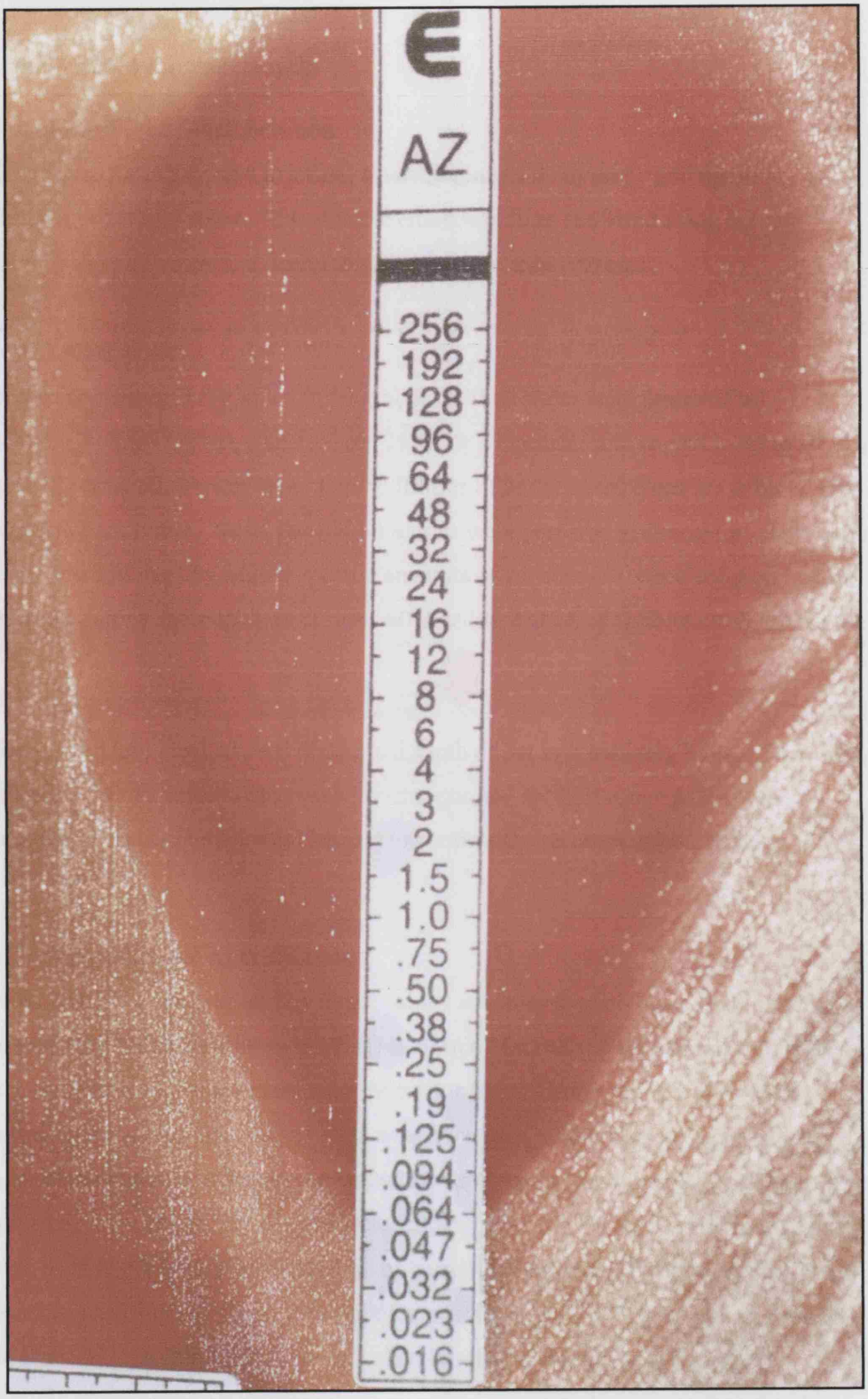


Figure 33: Azithromycin E test strip showing MIC of 0.064 µg/ml at the intersection of the lowest point of the elliptical zone of inhibition of bacterial growth with the E test strip

Agar Dilution Method for Vancomycin

Preparation of Antibiotic Stock Solution

Vancomycin (Sigma) was prepared to a final concentration of 8 mg/ml by adding 80 mg of antibiotic to 10 ml of sterile water. This stock solution was filter sterilized using 0.2 mm syringe filters (Nalgene Europe Ltd, Hereford, UK) into a sterile universal.

Preparation of Agar plates

Fourteen bottles containing 3.8 g MHA in 100 ml of distilled water were prepared as recommended by NCCLS (2003), sterilized at 121°C for 15 minutes and cooled to 45 to 50°C in a thermostatically controlled water bath. Five millilitres of defibrinated lysed horse blood (5% v/v) was added to each bottle. Serial two-fold dilutions were prepared at concentrations ranging from 0.125 mg/l to 256 mg/l by adding specific amounts of vancomycin stock solution to each bottle of agar and mixing thoroughly to ensure uniform distribution of antibiotic throughout the agar.

The agar was poured into labelled petri dishes to a depth of 3-4 mm avoiding bubble formation on the agar surface. Both uninoculated vancomycin agar and antibiotic-free MHA were included as control plates. The pH was checked to confirm the recommended pH range of 7.2-7.4.

Preparation of Inoculum for Agar Dilution

Bacterial broth cultures were prepared by direct colony suspension of colonies from overnight CBA plates into sterile Mueller Hinton Broth (Oxoid) to a MacFarland standard of 0.5. Plate inoculation was performed using a multipoint inoculator (Mast Group, Bootle, Merseyside, UK). Each well in the multipoint seeding plate was inoculated with 500 µl of bacterial suspension. Positive controls of *Staph. aureus* NCTC 6571 and *S. oralis* NCTC 11427, and a negative control of uninoculated broth were included with each run. A sterile multipoint spot inoculator was used to carry approximately 1 µl of bacterial suspension from the seeding plate to the agar surface resulting in a final inoculum of approximately 10^4–10^5 cfu/spot. A growth control plate (vancomycin-free MHA) was inoculated first and, starting with the lowest concentration, the plates containing different antimicrobial concentrations were inoculated. A second growth control plate was inoculated last to ensure that no contamination or significant antimicrobial carry over occurred during inoculation.

Inoculated plates were incubated for 16-20 hours at 37 °C in an atmosphere containing 5% CO_2 and examined for growth. The MIC was recorded as the lowest concentration of antimicrobial

agent that completely inhibited growth not including a faint haze caused by the inoculum (Figure 34).

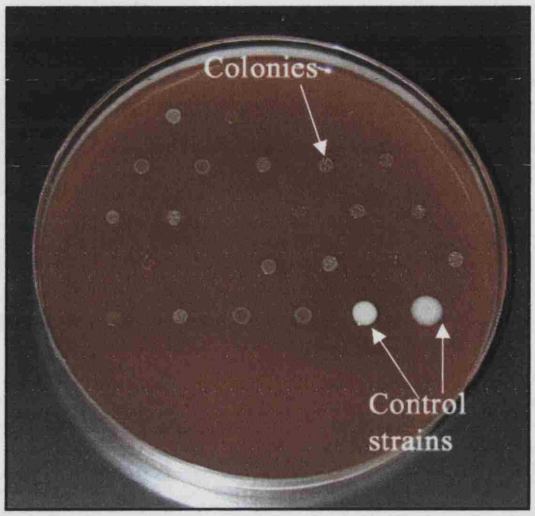


Figure 34: Streptococcal colonies on vancomycin MHA plate

Interpretation of Susceptibility Categories

Streptococci were classified as being susceptible, of intermediate susceptibility or resistant to each antibiotic according to the interpretive standards of the NCCLS (2003) for *Streptococcus* spp. as recommended by E test manufacturers (Table 43). E test breakpoints are based on a continuous scale while NCCLS breakpoints for antibiotics are based on broth microdilution susceptibility tests using double dilution techniques. The manufacturers recommend that E test MIC's which are between a conventional two-fold dilution are rounded up to the nearest higher two-fold dilution before susceptibility categorisation is carried out. Therefore E test MIC values (μg/ml) of 0.19, 0.38, 0.75, 1.5 and 3.0 were rounded up to 0.25, 0.5, 1.0, 2.0 and 4.0, respectively. The NCCLS does not provide categories for streptococci susceptibility to amoxicillin. For this reason breakpoints for ampicillin were used.

Table 43 : Breakpoints of Antibiotics for Streptococci According to the Interpretive Standards of the NCCLS (2003)

	Susceptible (MIC µg/ml)	Intermediate susceptibility (MIC µg/ml)	Resistant (MIC µg/ml)
Penicillin	≤ 0.12	0.25 – 2	≥ 4
Amoxicillin	≤ 0.25	0.5 – 4	≥8
Erythromycin	≤ 0.25	0.5	≥ 1
Azithromycin	≤ 0.5	1	≥ 2
Clarithromycin	≤ 0.25	0.5	≥ 1
Clindamycin	≤ 0.25	0.5	≥ 1
Cephalosporins	≤ 1	2	≥ 4
Vancomycin	≤ 1	-	-

Statistical Analysis

For each antibiotic the MIC_{50}, MIC_{90} and the minimum and maximum values were calculated. The proportions of susceptible, of intermediate susceptibility and resistant streptococci to each antibiotic were calculated for all the streptococci and for individual species. Spearman's rank correlation was used to investigate the association between the MIC's of oral streptococci to erythromycin with MIC's to clarithromycin and azithromycin. In addition, the correlation between the MIC's of the oral streptococci to penicillin with MIC's to amoxicillin, cephalothin, cephalexin and erythromycin were calculated in the same way.

RESULTS

Reproducibility

The type strains *S. oralis* NCTC 11427 and *Staph. aureus* NCTC 6571 was included in each run for the E test (n = 7) and vancomycin agar dilution (n = 3). The MIC values are shown in Table 44. The MIC values for the type strains of oral streptococci for the 11 antibiotics tested are shown in Table 45.

Table 44: MIC_{50} and MIC_{90} for *S. oralis* NCTC 11427 and *Staph. aureus* NCTC 6571

	S. oralis (n = 7)			*S. aureus* (n = 7)		
	MIC_{50}	**MIC_{90}**	**Min-Max**	**MIC_{50}**	**MIC_{90}**	**Min-Max**
Penicillin	0.032	0.16	0.016-0.16	0.032	0.047	0.023-0.047
Amoxicillin	0.023	0.16	0.016-0.16	0.047	0.064	0.047-0.064
Erythromycin	0.032	0.094	0.032-0.094	0.38	0.38	0.19-0.38
Azithromycin	0.023	0.32	0.016-0.32	0.75	0.75	0.75-1.0
Clarithromycin	0.032	0.094	0.023-0.094	0.25	0.5	0.19-0.5
Clindamycin	0.064	0.094	0.047-0.094	0.094	0.19	0.064-0.19
Gentamicin	24	32	16-32	0.75	0.75	0.19-0.75
Cephalexin	2	3	2-3	0.094	0.094	0.047-0.094
Cephalothin	0.19	0.25	0.19-0.25	0.75	1.0	0.75-1.0
Teicoplanin	0.125	0.125	0.094-0.125	1.5	3.0	1.0-3.0
Vancomycin	1.0	1.5	1-1.5	3.0	4.0	3.0-4.0
Van (AgD)*	0.5	0.5	0.5	1.0	1.0	1.0-2.0

n = number of times reproducibility was carried out using the E test

*Vancomycin susceptibility testing using agar dilution technique was performed on 3 occasions

Table 45: MIC Values (µg/ml) for Streptococcal Type Strains to the Antibiotics Tested

	S. mitis NCTC 12261	*S. gordonii* NCTC 7865	*S. salivarius* NCTC 8618	*S. sobrinus* NCTC 12279	*S. mutans* NCTC 10449	*S. sanguinis* NCTC 7863	*S. parasanguinis* NCTC 55898	*S. anginosus* NCTC 10713	*S. pneumoniae* NCTC 12166
Penicillin	0.016	0.032	0.047	0.016	0.023	0.19	0.094	0.094	0.016
Amoxicillin	0.023	0.125	0.032	0.032	0.032	0.25	0.19	0.064	0.032
Erythromycin	0.094	0.094	0.19	0.023	0.125	0.047	0.064	0.064	0.032
Azithromycin	0.19	0.125	0.5	0.032	0.125	0.064	0.064	0.064	0.032
Clarithromycin	0.125	0.047	0.094	0.016	0.047	0.032	0.032	0.032	0.016
Clindamycin	0.064	0.125	0.19	0.032	0.064	0.094	0.064	0.064	0.032
Gentamicin	1.5	4	8	3	1.5	3	6	6	4
Cephalexin	3	6	3	0.75	0.75	24	2	2	0.75
Cephalothin	0.25	0.5	0.125	0.25	0.19	0.38	0.19	0.38	0.38
Teicoplanin	0.94	0.125	0.25	0.5	0.5	0.25	0.125	0.25	0.064
Vancomycin	1.5	1.5	2	1.5	3	1	2	1.5	0.5
Van (AgD)*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

*Vancomycin susceptibility testing using agar dilution technique

Streptococci Isolates from Blood Samples

The MIC frequency distribution, MIC_{50}, MIC_{90} and range of the 63 streptococci for each antibiotic are shown in Table 46 andTable 47. The number and proportion of streptococci susceptible, of intermediate susceptibility and resistant to each antibiotic are shown in Table 48. Susceptibility breakpoints for gentamicin and teicoplanin are not provided by NCCLS and susceptibility categorization could not be carried out for them. Vancomycin susceptibility using both the E test method and agar dilution are shown in Table 46, Table 47 and Table 48.

Penicillin

Forty-two streptococcal isolates (67%) were susceptible to penicillin. Intermediate susceptibility (MIC = 0.25–2 μg/ml) was observed in 19 isolates (30%) and two isolates (3%) were resistant (MIC ≥ 4 μg/ml).

Amoxicillin

Fifty isolates (79%) were susceptible to amoxicillin, 12 isolates (19%) were of intermediate susceptibility (MIC = 0.5–4.0 μg/ml) and one isolate (2%) was resistant (MIC ≥ 8.0 μg/ml).

Erythromycin

Forty-three isolates (68%) were susceptible to erythromycin, one (2%) was of intermediate susceptibility (MIC = 0.5 μg/ml) and 19 isolates (30%) were resistant (MIC ≥ 1.0 μg/ml).

Azithromycin

Forty-three isolates (68.3%) were susceptible to azithromycin, two isolates (3.2%) were of intermediate susceptibility (MIC 1.0 μg/ml) and 18 isolates (28.5%) were resistant (MIC ≥ 2.0 μg/ml).

Clarithromycin

Forty-two isolates (66.7%) were susceptible to clarithromycin, three isolates (4.8%) were of intermediate susceptibility (MIC = 0.5 μg/ml), and 18 isolates (28.5%) were highly resistant (MIC ≥ 2.0 μg/ml).

Clindamycin

Fifty-four isolates (85.7%) were susceptible to clindamycin (MIC $\leq$ 0.25 μg/ml). Three isolates (4.8%) were of intermediate susceptibility (MIC = 0.5 μg/ml) and six isolates (9.5%) were resistant (MIC $\geq$ 1.0 μg/ml).

Cephalexin

Only eight isolates (13%) were susceptible to cephalexin (MIC $\leq$ 1 μg/ml). Twenty-two isolates (35%) were of intermediate susceptibility (MIC 2.0 μg/ml) and 33 (52%) were resistant (MIC $\geq$ 4.0 μg/ml).

Cephalothin

Fifty-six isolates (89%) were susceptible to cephalothin (MIC $\leq$ 1 μg/ml). Four isolates (6%) were of intermediate susceptibility (MIC 2.0 μg/ml) and three isolates (5%) were resistant (MIC $\geq$ 4.0 μg/ml).

Gentamicin

The MIC's of 54 isolates (86%) were equal to or above 3 μg/ml. Susceptibility breakpoints for gentamicin are not provided by the NCCLS and susceptibility categorization could not be performed.

Teicoplanin

The MIC's of 50 of the isolates (79%) were in the range of 0.09–0.25 μg/ml. Susceptibility breakpoints for teicoplanin are not provided by the NCCLS and susceptibility categorization could not be performed.

Vancomycin

Only 11 isolates (17.5%) were susceptible (MIC $\leq$ 1.0 μg/ml) to vancomycin using the E test and 52 isolates (82.5%) were resistant.

Vancomycin – agar dilution

All 63 isolates were susceptible to vancomycin using the agar dilution technique.

Table 46: Frequency Distribution of Antimicrobial Sensitivity for Streptococci Isolated from Blood Samples

	Number of streptococci clinical isolates for which the MIC (µg/ml) was:																									
	0.016	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**96**	**128**	**256**
PG	6	6	9	3	6	4	8	4	5	-	1	4	4	-	1	2	-	-	-	-	-	-	-	-	-	-	-
AC	3	3	4	9	3	5	6	6	7	4	2	1	4	1	3	1	-	-	1	-	-	-	-	-	-	-	-
EM	5	7	4	9	6	8	3	1	-	1	-	2	-	1	4	3	3	1	1	3	-	-	-	-	-	-	1
AZ	4	2	1	5	5	10	5	4	2	4	1	-	2	1	2	2	2	3	4	-	-	1	-	-	1	1	1
CH	10	9	6	8	3	4	-	2	-	2	1	-	2	2	4	5	1	1	1	-	-	-	-	1	-	-	1
CM	3	1	3	6	10	15	5	9	2	2	1	-	-	-	2	1	-	-	-	-	-	-	-	-	-	1	2
GM	-	-	-	-	1	-	-	1	1	1	-	-	1	2	2	6	14	12	4	4	7	4	-	3	-	-	-
CX	1	-	-	-	-	-	1	-	-	4	1	1	-	-	17	5	1	6	8	5	2	4	4	1	-	-	2
CE	1	-	1	2	1	-	-	5	10	10	10	6	10	2	2	-	2	-	1	-	-	-	-	-	-	-	-
TP	-	-	-	1	-	6	17	21	6	2	4	1	2	1	1	-	-	1	-	-	-	-	-	-	-	-	-
VA	-	-	-	-	-	-	-	-	-	-	3	2	6	25	18	9	-	-	-	-	-	-	-	-	-	-	-
V(AgD)*	-	-	-	-	-	-	2	-	13	-	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Vancomycin susceptibility testing using agar dilution technique

PG = penicillin, AC = amoxicillin, EM = erythromycin, AZ = azithromycin, CH = clarithromycin, CM = clindamycin, GM = gentamicin, CX = cephalexin, CE = cephalothin, TP = teicoplanin, VA = vancomycin

Table 47: MIC_{50} and MIC_{90} Values for Streptococcal Clinical Isolates

(n = 63)	MIC_{50}	MIC_{90}	Range
Penicillin	0.094	1	≤0.016 – 3
Amoxicillin	0.125	1	≤0.016 – 8
Erythromycin	0.094	6	0.016 – 256
Azithromycin	0.125	8	0.016 – 256
Clarithromycin	0.047	2	0.016 – 256
Clindamycin	0.094	0.5	0.016 – 256
Gentamicin	6	24	0.064 – 48
Cephalexin	3	24	0.016 – 256
Cephalothin	0.5	1.5	0.016 – 8
Teicoplanin	0.19	0.5	0.094 – 6
Vancomycin	1.5	3	0.5 – 3
Vancomycin - (AgD)*	0.5	0.5	0.125 – 0.5

n = number of isolates

*Vancomycin susceptibility testing using agar dilution technique

Table 48: Susceptibility, Intermediate Susceptibility and Resistance of Streptococcal Clinical Isolates

(n = 63)	Susceptible		Intermediate susceptibility		Resistant	
	n	%	n	%	n	%
Penicillin	42	67	19	30	2	3
Amoxicillin	50	79	12	19	1	2
Erythromycin	43	68	1	2	19	30
Azithromycin	43	68.3	2	3.2	18	28.5
Clarithromycin	42	66.7	3	4.8	18	28.5
Clindamycin	54	85.7	3	4.8	6	9.5
Cephalexin	8	13	22	35	33	52
Cephalothin	56	89	4	6	3	5
Vancomycin	11	17.5	-	-	52	82.5
Vancomycin - (AgD)*	63	100	-	-	0	0

n = number of isolates

*Vancomycin susceptibility testing using agar dilution technique

Correlation Between the MIC's of Erythromycin and Clarithromycin and Azithromycin

There was a high correlation between the MIC's of erythromycin and azithromycin and erythromycin and clarithromycin for all streptococci tested (Table 49).

Table 49: Correlation Between MIC's of Erythromycin versus Azithromycin and Clarithromycin

		Clarithromycin	Azithromycin
Erythromycin	Rho	0.87	0.98
	p-value	$p \leq 0.0001$	$p \leq 0.0001$

rho = Spearman's rank correlation

Correlation Between MIC's of Penicillin and Amoxicillin, Cephalothin, Cephalexin and Erythromycin

There was a high correlation between the MIC's of penicillin and amoxicillin, cephalothin and cephalexin ($p \leq 0.0001$). There was no correlation between MIC's of penicillin and those of erythromycin (Table 50).

Table 50: Correlation Between MIC's of Penicillin versus Amoxicillin, Cephalothin, Cephalexin and Erythromycin

		Amoxicillin	Cephalothin	Cephalexin	Erythromycin
Penicillin	Rho	0.87	0.78	0.51	0.027
	p value	$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.0001$	$p \leq 0.83$

rho = Spearman's rank correlation

Antibiotic Sensitivity of Each *Streptococcus* Species

S. oralis

The MIC's for *S. oralis* (n = 24) are shown in Table 51. All isolates were susceptible to penicillin and amoxicillin. Susceptibility to erythromycin, azithromycin and clarithromycin was lower, 63%, 63% and 67%, respectively. Twenty-one isolates (88%) were susceptible to clindamycin and all were susceptible to cephalothin. Thirteen isolates (54%) were susceptible to cephalexin. Only seven isolates (29%) were susceptible to vancomycin using the E test. All the isolates were susceptible using the agar dilution technique.

S. mitis

The MIC's for *S. mitis* (n = 8) are shown in Table 51. All eight isolates were susceptible to penicillin, amoxicillin, clindamycin and cephalothin. Six isolates (75%) were susceptible to erythromycin, azithromycin and clarithromycin but only two (25%) were of intermediate susceptibility to cephalexin. Only one isolate (13%) was susceptible using the E test. All isolates were susceptible to vancomycin using the agar dilution technique.

S. gordonii

The MIC's for *S. gordonii* (n = 7) are shown in Table 51. All seven isolates were susceptible to penicillin, amoxicillin, clindamycin and cephalothin. Six isolates (86%) were susceptible to erythromycin and azithromycin and five (71%) were susceptible to clarithromycin. Only two (29%) isolates were of intermediate susceptibility to cephalexin. Only one (14%) was susceptible by the E test method. All isolates were susceptible to vancomycin using the agar dilution technique.

S. sanguinis

The MIC's for *S. sanguinis* (n = 5) are shown in Table 51. Four (80%) isolates were susceptible to penicillin, amoxicillin, erythromycin, azithromycin, clarithromycin and clindamycin. Three isolates (60%) were susceptible to cephalothin. All isolates were resistant to cephalexin. All isolates were resistant to vancomycin using the E test, while all isolates were susceptible using the agar dilution technique.

S. parasanguinis

The MIC's for *S. parasanguinis* (n = 4) are shown in Table 51. All four isolates were susceptible to penicillin, amoxicillin, clindamycin and cephalothin. Three isolates (75%) were susceptible to erythromycin, azithromycin and clarithromycin and one isolate was of intermediate susceptibility to cephalexin. All isolates were resistant to vancomycin using the E test, while all isolates were susceptible using the agar dilution technique.

S. pneumoniae

The MIC's for *S. pneumoniae* (n = 4) are shown in Table 51. All isolates were susceptible to amoxicillin and clindamycin. Three isolates (75%) were susceptible to penicillin, erythromycin, azithromycin, clarithromycin and cephalothin. Two isolates (50%) were susceptible to cephalexin. Only one isolate (25%) was susceptible to vancomycin using the E test method, while all four isolates were susceptible using the agar dilution technique.

S. infantis

The MIC's for individual isolates of *S. infantis* (n = 3) are shown in Table 52. All three isolates were susceptible to penicillin, amoxicillin and cephalothin. One isolate was resistant to erythromycin and azithromycin and of intermediate susceptibility to clarithromycin. Two isolates were susceptible to clindamycin and one was of intermediate susceptibility. Two isolates were resistant to cephalexin. All isolates were resistant to vancomycin using the E test, while all isolates were susceptible using the agar dilution technique.

S. australis

The MIC's for individual isolates of *S. australis* (n = 2) are shown in Table 53. Two isolates were resistant to cephalexin. One isolate was resistant to erythromycin, azithromycin and clarithromycin.

S. peroris

The MIC's for individual isolates of *S. peroris* (n = 2) are shown in Table 53. Both isolates were resistant erythromycin, azithromycin and clarithromycin. One isolate was resistant to clindamycin and cephalexin.

S. constellatus

The MIC's for individual isolates of *S. constellatus* (n = 2) are shown in Table 53. Both isolates were susceptible to all the antibiotics, except for vancomycin using the E test method.

Table 51: Range, MIC_{50} and MIC_{90} for Each *Streptococcus* sp. and Proportion Susceptible to the Antibiotics Tested

Species	Antibiotic	MIC (μg/ml)			S% (S and I %)
		Range	50	90	
S. oralis	Penicillin	0.016-2.0	0.064	0.75	75 (100)
(n = 24)	Amoxicillin	0.02-3.0	0.13	1.0	88 (100)
	Erythromycin	0-12	0.094	4.0	63
	Azithromycin	0-128	0.125	6.0	54 (63)
	Clarithromycin	0-8	0.064	3.0	63 (67)
	Clindamycin	0-3	0.094	2.0	83 (88)
	Gentamicin	0-48	12	24	NA
	Cephalexin	0-32	2.0	24	21 (54)
	Cephalothin	0-1	0.38	1.0	100
	Teicoplanin	0.13-2.0	0.19	0.5	NA
	Vancomycin	1-3	1.5	3.0	29
	Van (AgD)*	0.25-0.5	0.5	0.5	100
S. mitis	Penicillin	0.032-1.0	0.125	1.0	50 (100)
(n = 8)	Amoxicillin	0.047-2.0	0.125	2.0	88 (100)
	Erythromycin	0.032-3.0	0.064	3.0	75
	Azithromycin	0.064-8.0	0.094	8.0	50 (75)
	Clarithromycin	0.023-2.0	0.032	2.0	75
	Clindamycin	0.047-0.19	0.094	0.19	100
	Gentamicin	1.0-48.0	3.0	48.0	NA
	Cephalexin	2.0-48.0	8.0	48.0	0 (25)
	Cephalothin	0.19-2.0	0.5	2.0	75 (100)
	Teicoplanin	0.047-0.19	0.094	0.19	NA
	Vancomycin	1.0-2.0	1.5	2.0	13
	Van (AgD)*	0.25-0.5	0.5	0.5	100
S. gordonii	Penicillin	0.016-0.5	0.032	0.5	86 (100)
(n = 7)	Amoxicillin	0.016-0.38	0.094	0.38	100
	Erythromycin	0.032-12.0	0.047	12.0	86
	Azithromycin	0.047-8.0	0.094	8.0	86
	Clarithromycin	0.016-48.0	0.032	48.0	71
	Clindamycin	0.094-0.25	0.19	0.25	100
	Gentamicin	4.0-16.0	6.0	16.0	NA
	Cephalexin	2.0-32.0	3.0	32.0	0 (29)
	Cephalothin	0.25-2.0	0.5	2.0	86 (100)
	Teicoplanin	0.125-1.0	0.19	1.0	NA
	Vancomycin	0.5-2.0	1.5	2.0	14
	Van (AgD)*	0.125-0.5	0.5	0.5	100

n = number of isolates
*Vancomycin susceptibility testing using agar dilution technique
S = susceptible; I = intermediate susceptibility (MIC = μg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS

Table 51: Range, MIC_{50} and MIC_{90} for Each *Streptococcus* sp. and Proportion Susceptible to the Antibiotics Tested (cont.)

Species	Antibiotic	MIC (µg/ml)			S% (S and I %)
		Range	50	90	
S. sanguinis	Penicillin	0.094-3.0	0.125	3.0	60 (80)
(n = 5)	Amoxicillin	0.25-8.0	0.5	4.0	40 (80)
	Erythromycin	0.016-4.0	0.023	4.0	80
	Azithromycin	0.016-4.0	0.125	96.0	80
	Clarithromycin	0.016-1.5	0.023	1.5	80
	Clindamycin	0.016-192	0.047	192	80
	Gentamicin	3.0-8.0	6.0	8.0	NA
	Cephalexin	4.0-256	16.0	256	0
	Cephalothin	0.38-4.0	1.0	4.0	60
	Teicoplanin	0.125-0.19	0.19	0.19	NA
	Vancomycin	1.5-2.0	1.5	2.0	0
	Van (AgD)*	0.5	0.5	0.5	100
S. parasanguinis	Penicillin	0.25-0.75	0.75	0.75	100
(n = 4)	Amoxicillin	0.032-1.0	1.0	1.0	100
	Erythromycin	0.023-8.0	0.064	8.0	75
	Azithromycin	0.032-3.0	0.094	3.0	75
	Clarithromycin	0.023-3.0	3.0	3.0	75
	Clindamycin	0.032-0.094	0.047	0.094	100
	Gentamicin	3.0-6.0	4.0	6.0	NA
	Cephalexin	2.0-12.0	6.0	12.0	0 (25)
	Cephalothin	0.19-0.38	0.5	1.0	100
	Teicoplanin	0.19-0.38	0.25	0.38	NA
	Vancomycin	1.5-2.0	1.5	2.0	0
	Van (AgD)*	0.5	0.5	0.5	100
S. pneumoniae	Penicillin	0.016-3.0	0.016	3.0	75
(n = 4)	Amoxicillin	0.016-2.0	0.023	2.0	75 (100)
	Erythromycin	0.016-4.0	0.094	4.0	75
	Azithromycin	0.016-8.0	0.19	8.0	75
	Clarithromycin	0.016-3.0	0.047	3.0	75
	Clindamycin	0.016-0.19	0.094	0.19	100
	Gentamicin	0.25-6.0	4.0	6.0	NA
	Cephalexin	0.016-256	2.0	256	25 (50)
	Cephalothin	0.094-0.38	0.25	8.0	75
	Teicoplanin	0.25-0.5	0.094	0.38	NA
	Vancomycin	1.0-2.0	1.5	2.0	25
	Van (AgD)*	0.25-0.5	0.5	0.5	100

n = number of isolates
*Vancomycin susceptibility testing using agar dilution technique
S = susceptible; I = intermediate susceptibility (MIC = µg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS

Table 52: MIC Values and Susceptibility of *S. infantis* to the Antibiotics Tested

	S. infantis (n = 3)	
	MIC	**Susceptibility**
Penicillin	0.016	S
	0.094	S
	0.125	S
Amoxicillin	0.016	S
	0.094	S
	0.125	S
Erythromycin	0.016	S
	0.38	I
	1.5	R
Azithromycin	0.023	S
	1.0	I
	1.5	R
Clarithromycin	0.016	S
	0.38	I
	0.5	I
Clindamycin	0.047	S
	0.064	S
	0.5	I
Gentamicin	3.0	NA
	6.0	NA
	6.0	NA
Cephalexin	0.75	S
	12.0	R
	16.0	R
Cephalothin	0.047	S
	0.5	S
	0.75	S
Teicoplanin	0.094	NA
	0.19	NA
	1.0	NA
Vancomycin	1.5	R
	1.5	R
	3.0	R
Vancomycin (AgD)*	0.5	S
	0.5	S
	0.5	S

S = susceptible; I = intermediate susceptibility, R = resistant; (MIC = µg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS
*Vancomycin susceptibility testing using agar dilution technique

Table 53: MIC Values and Susceptibility of *S. australis*, *S. peroris* and *S. constellatus* to the Antibiotics Tested

	S. australis (n =2)		*S. peroris* (n = 2)		*S. constellatus* (n = 2)	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**	**MIC**	**Susceptibility**
Penicillin	0.125	S	0.032	S	0.016	S
	1.0	I	0.125	S	0.064	S
Amoxicillin	0.19	S	0.094	S	0.032	S
	0.75	S	0.25	S	0.125	S
Erythromycin	0.064	S	2	R	0.047	S
	0.75	R	6	R	0.094	S
Azithromycin	0.19	S	24	R	0.094	S
	0.75	R	256	R	0.19	S
Clarithromycin	0.032	S	6	R	0.032	S
	1.0	R	256	R	0.064	S
Clindamycin	0.064	S	0.094	S	0.047	S
	0.38	S	256	R	0.125	S
Gentamicin	0.19	NA	8	NA	3.0	NA
	4.0	NA	12	NA	6.0	NA
Cephalexin	3.0	R	2	I	2.0	I
	12.0	R	6	R	2.0	I
Cephalothin	0.25	S	0.25	S	0.19	S
	0.38	S	1.0	S	0.25	S
Teicoplanin	0.19	NA	0.125	NA	0.125	NA
	6.0	NA	1.0	NA	0.125	NA
Vancomycin	1.0	S	2.0	R	2.0	R
	3.0	R	3.0	R	3.0	R
Van (AgD)*	0.25	S	0.5	S	0.5	S
	0.5	S	0.5	S	0.5	S

S = susceptible; I = intermediate susceptibility, R = resistant; (MIC = μg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS
*Vancomycin susceptibility testing using agar dilution technique

S. salivarius and S. mutans

The MIC's for individual isolates of *S. salivarius* (n = 1) and *S. mutans* (n = 1) are shown in Table 54. *S. salivarius* was resistant to cephalexin and to vancomycin using the E test. *S. mutans* was susceptible to all the antibiotics, except for vancomycin using the E test.

Table 54: MIC Values and Susceptibility of *S. salivarius* and *S. mutans* to the Antibiotics Tested

	S. salivarius (n = 1)		*S. mutans* (n = 1)	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Penicillin	1	S	0.023	S
Amoxicillin	0.25	S	0.032	S
Erythromycin	0.064	S	0.064	S
Azithromycin	0.125	S	0.047	S
Clarithromycin	0.047	S	0.023	S
Clindamycin	0.047	S	0.064	S
Gentamicin	3.0	NA	4.0	NA
Cephalexin	8.0	R	0.38	S
Cephalothin	1.5	I	0.064	S
Teicoplanin	0.75	NA	1.5	NA
Vancomycin	2.0	R	3.0	R
Van (AgD)*	0.5	S	0.5	S

S = susceptible; I = intermediate susceptibility, R = resistant; (MIC = μg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS
*Vancomycin susceptibility testing using agar dilution technique

DISCUSSION

Resistance to ß-lactams has developed within a wide range of bacteria since the introduction of penicillin in the 1940's. Emergence of antibiotic-resistant strains of oral streptococci is a cause of concern and could compromise the effectiveness of currently used endocarditis prophylaxis and therapeutic regimens (Carratalá et al 1995; Doern et al 1996).

The E test has been found to compare favourably with both agar dilution and broth dilution methods in measuring antimicrobial MIC's for a variety of Gram-positive organisms, including staphylococci (Weller et al 1997), *Streptococcus pneumoniae* (Tenover et al 1996), nutritionally variant streptococci (Douglas et al 1994) and streptococci isolated from blood (Renneberg et al 1997; Rosser et al 1999). It is not the most reliable method for testing the susceptibility of oral streptococci to vancomycin. This is because the MIC values have been found to be falsely elevated (Rosser et al 1999). The E test MIC's of vancomycin were 1.5 dilutions greater than the corresponding agar dilution MIC's. Different workers have reported elevated E test MIC values for vancomycin for 37 clinical isolates of *S. pneumoniae* and the type strain ATCC 49619 (Hashemi et al 1996). The reason for this discrepancy was believed to be related to the organism tested, rather than to the test itself, as control strains of *Staph. aureus* gave good agreement (Hashemi et al 1996; Rosser et al 1999).

In the present study 52 isolates (82.5%) were resistant to vancomycin (MIC > 1 μg/ml) using the E test. For this reason antibiotic sensitivity testing for vancomycin was repeated using the agar dilution method and all isolates were found to be susceptible. This is in agreement with results of other researchers (Teng et al 1998; Wisplinghoff et al 1999; Marron et al 2001; Kennedy et al 2001). Higher MIC values of vancomycin with the E test compared to standard agar or broth dilution methods have been reported when testing susceptibility of Gram-positive bacteria (Baker et al 1991), staphylococci (Huang et al 1992), enterococci (Tenover et al 1995) and coryneform bacteria (Martínez-Martínez et al 1995).

Antibiotic sensitivity testing of samples from the saliva, gingival crevice and gingival margin of healthy patients attending Cardiff Dental Hospital revealed penicillin-resistant oral bacteria in 85% of the subjects. Twenty-five percent of the patients harboured penicillin-resistant streptococci (Drucker & Jolly 1971). More recently, 97% of a group of healthy children were found to have penicillin-resistant oral organisms (Ready et al 2003). Of these, 27% of *Streptococcus* spp. were resistant to ampicillin.

High level resistance to penicillin has been reported in 13% of 'viridans' group streptococci isolated from blood from Canadian hospitalized patients (Rosser et al 1999) and 5.6% of

American inpatients (Doern et al 1996). Other workers reported that 2% of streptococci isolated from the blood of neutropenic cancer patients were of high level resistance and 19% were of intermediate resistance (Wisplinghoff et al 1999). This is similar to results in the present study in which high level resistance to penicillin (MIC $\geq$ 4 μg/ml) was observed in only two isolates (3%) but 30% of streptococci isolated were of intermediate susceptibility (MIC 0.25 – 2 μg/ml).

Macrolides and cephalosporins have been suggested as alternative prophylaxis for IE. Erythromycin A resistance has spread worldwide since the first report of decreased erythromycin susceptibility of *S. pyogenes* in 1959 (Lowbury & Hurst 1959). In Spain, workers examining pharyngeal exudates found that 61% of 'viridans' group streptococci isolated from 98 outpatients and 51% from 50 healthy volunteers were resistant to erythromycin A (Aracil et al 2001). Researchers in the UK reported resistance to erythromycin in 59% of *Streptococcus* spp. isolated from saliva of healthy children (Ready et al 2003). A study investigating the prevalence of antibiotic resistance in streptococci causing bacteraemia in neutropenic cancer patients revealed that 36% of isolates were resistant to erythromycin (Marron et al 2001).

Recently, 23% of 'viridans' streptococci isolated from orofacial infections were found to be resistant to erythromycin (Kuriyama et al 2002). Thirty percent of isolates in the study reported here had high level resistance to erythromycin and 2% had intermediate level resistance. In addition, the two other macrolide antibiotics recommended for antibiotic prophylaxis, clarithromycin and azithromycin, exhibited high level resistance in 28.5% of isolates. This is in agreement with earlier work (Teng et al 1998; Marron et al 2001). The MIC's showed significant correlation with those of erythromycin.

Azithromycin has been reported to be superior to ampicillin for prophylaxis against streptococci, with 94% and 72% efficacy, respectively (Tsitsika et al 2000). This highlights a significant problem in the choice of alternatives for penicillin allergic individuals. In a different investigation, 32% of streptococci isolated from the blood of neutropenic cancer patients were resistant to erythromycin but only 2% were resistant to penicillin (Wisplinghoff et al 1999).

Cephalothin was used in this investigation instead of cephazolin because E test strips are not manufactured for cephazolin. It is of note that cephazolin and cephalexin are recommended by the American Heart Association for prophylaxis against IE (Dajani et al 1997). Of the streptococcal isolates tested, 52% were highly resistant to cephalexin but only 5% to cephalothin. Other workers investigating 'viridans' group streptococci isolated from blood found that 96% were highly resistant to cephalexin. Fifteen to twenty percent were resistant to

cefprozil, cefuroxime, cefpodoxime, and ceftriaxone (Doern et al 1996). Similarly, different levels of resistance to four different cephalosporins have been reported in oral streptococci causing bacteraemia in neutropenic cancer patients (Marron et al 2001). Twenty-two percent were resistant to ceftriaxone, 53% to ceftazidime, 14% to cefpirome and 12% to cefepime.

Out of five IE cases caused by oral streptococci with high level resistance to penicillin and third generation cephalosporins, four resolved only after a prolonged course of glycopeptides (Levitz 1999; Lonks et al 1999; Levy et al 2001; Sabella et al 2001). The glycopeptides, vancomycin and teicoplanin, are recommended as alternatives for penicillin allergic and high risk patients (Roberts et al 2004; Horstkotte et al 2004). Acquired resistance to these antibiotics is rare, although vancomycin resistant strains of enterococci (Rosenberg et al 2004) and staphylococci (Biavasco et al 2000) have emerged and are causing concern.

Teicoplanin is an alternative to vancomycin with a similar spectrum of activity and mechanism of action. Unlike vancomycin, teicoplanin has a prolonged half life allowing once daily administration, is well tolerated intramuscularly and has a less toxic profile (Campoli-Richards et al 1990). There have been better results using teicoplanin in the prevention of experimental IE due to *S. oralis*, *E. faecium* and methicillin-resistant *Staph. aureus* compared to vancomycin (Perdikaris et al 1997). In the present investigation, 50% of streptococcal isolates were inhibited by teicoplanin at 0.19 μg/ml (MIC_{50}) and 90% were inhibited at 0.5 μg/ml (MIC_{90}). These are similar to the results obtained by other researchers (Renneberg et al 1997; Teng et al 1998; Wisplinghoff et al 1999).

A variation in antibacterial resistance within different streptococcal species has been demonstrated. Bacteria isolated from confirmed cases of IE in the UK were tested for penicillin resistance. Eighty-six percent of the isolates were oral streptococci. Of these, 15% of *S. sanguinis*, 13% of *S. oralis* and 6% of *S. gordonii* had reduced susceptibility to penicillin. All *S. bovis* and *S. mutans* isolates were susceptible to penicillin (Johnson et al 2001). This is in agreement with the findings reported here in which 20% of *S. sanguinis* isolates had high level resistance to penicillin and amoxicillin. In Taiwan, *S. oralis* was found to have the most frequent high level resistance to penicillin and the macrolide antibiotics (Teng et al 1998). Other workers reported the greatest percentage of high level resistance to penicillin with *S. salivarius*, *S. mitis* and *S. oralis*. They also found that the species with the lowest proportion of full susceptibility (65%) and the highest level of intermediate susceptibility (35%) was *S. sanguinis* (Rosser et al 1999). A greater proportion of *S. salivarius* with penicillin resistance (17%) than *S. mitis* (16%) and *S. sanguinis* (9%) has been reported (Doern et al 1996). The API 20 Strep system was used to identify the oral streptococci in these studies, which is less reliable

than the API ID 32 Strep system (Kikuchi et al 1995). Species identification of the streptococci in the current study was by *sodA* gene sequencing. This is an established method for streptococci identification, particularly in differentiating the mitis group (Kawamura et al 1999). This may explain the slight differences in the predominance of resistant species between this study and other studies.

In the study reported here, all five isolates of *S. sanguinis* were highly resistant to cephalexin and two were also resistant to cephalothin. Three out of four isolates of *S. parasanguinis* were also highly resistant to cephalexin but all were susceptible to cephalothin. Forty-six percent of *S. oralis* were highly resistant to cephalexin and six out of the eight *S. mitis* isolates were highly resistant. Macrolide resistance was generally the same amongst the species with 37% of *S. oralis*, 25% of *S. mitis*, 20% of *S. sanguinis*, 25% of *S. parasanguinis* and 25% of *S. pneumoniae* with high level resistance.

Summary

In the current investigation, *S. oralis*, *S. mitis* and *S. sanguinis* had the highest levels of resistance to some of the antibiotics which are recommended for endocarditis prophylaxis. This is in agreement with earlier work (Doern et al 1996; Teng et al 1998; Rosser et al 1999). These oral streptococcal species are also important in the aetiology of IE (Douglas et al 1993; Larsen et al 1999; Hoen et al 2002). It is important to reassess the routine use of cephalexin and the macrolides (azithromycin and clarithromycin) for antibiotic prophylaxis in at risk patients.

CHAPTER 9
ANTIBIOTIC SENSITIVITY OF COAGULASE-NEGATIVE STAPHYLOCOCCI AND OTHER BACTERIA

INTRODUCTION

CNS are increasing in importance as causes of bacteraemia and IE (Etienne & Eykyn 1990; Rupp & Archer 1994; Huebner & Goldmann 1999). Prosthetic valve IE is caused by CNS in 40 to 50% of cases (Karchmer 1999). An increase in native valve IE caused by CNS has emerged presenting with acute infection requiring valve replacement (Etienne & Eykyn 1990; Miele et al 2001). The National Committee of Infection Survey (NCIS) found that from 1980 to 1989 the prevalence of CNS as a cause of nosocomial bacteraemia increased from 9% to 27% to become the single most common cause of these infections (Schaberg et al 1991). Data revealed that, during the same period, the proportion of nosocomial CNS resistant to methicillin and oxacillin increased from 20% to 60% (Schaberg et al 1991). Most of these resistant CNS were resistant to multiple additional antimicrobial agents. This confirmed an association between the dramatic increase in CNS as a cause of nosocomial bacteraemia and the resistance of these pathogens to antimicrobial agents.

At present, more than 70% of CNS isolates worldwide are resistant to oxacillin (Diekema et al 2001; Fritsche et al 2003). Erythromycin resistance has been reported in 71% of CNS isolates (Fritsche et al 2003). Recently, high resistance rates by CNS collected from 30 centres in the UK to most of the antibiotics tested have been reported (Johnson et al 2003). These included oxacillin (78.5%), erythromycin (61.9%), gentamicin (67.6%), ciprofloxacin (38.5%) and teicoplanin (13.7%). Resistance to vancomycin was seen in six isolates (1.2%) (Johnson et al 2003). As a result of the emergence of CNS resistant to a range of antibiotics, it is important to assess and report their susceptibility to the antibiotics recommended for IE prophylaxis.

I. ANTIBIOTIC SENSITIVITY OF COAGULASE-NEGATIVE STAPHYLOCOCCI

Materials and Methods:

Antibiotic sensitivity of CNS was determined using E test strips (AB Biodisk) for the same antibiotics described in Chapter 8. Cephazolin was replaced with cephalothin as no E test strips are available for cephazolin.

Type Strains

The type strain included in each batch was *Staph. aureus* NCTC 6571. Antibiotic sensitivity testing was carried for the following type strains: *Staph. epidermidis* NCTC 11047, *Staph. hominis* NCTC 11320, *Staph. warneri* NCTC 10518, *Staph. capitis* NCTC 11045, *Staph. cohnii* NCTC 10515 and *Staph. saprophyticus* NCTC 10516.

E test Method

The same method was used as for the oral streptococci described in Chapter 8. Eighty-nine CNS clinical isolates were tested. These were *Staph. epidermidis* (n = 28), *Staph. hominis* (n = 44), *Staph.warneri* (n = 9), *Staph. capitis* (n = 3), *Staph. cohnii* (n =3) and *Staph. saprophyticus* (n = 2).

Vancomycin Agar Dilution

Vancomycin susceptibility was determined by the agar dilution technique as described in the previous chapter.

Reproducibility

Susceptibility to the antibiotics was repeated on nine occasions for the reference strain of *Staph. aureus* NCTC 6571 to check for reproducibility of the E test method and on four occasions for vancomycin using the agar dilution technique.

Interpretation of Susceptibility Categories

CNS were classified as being susceptible, of intermediate susceptibility or resistant to each antibiotic according to the interpretive standards of the NCCLS (2003) for *Staphylococcus* spp. (Table 55), as recommended by the E test manufacturers.

Table 55: Breakpoints of Antibiotics for *Staphylococcus* spp. According to the Interpretive Standards of the NCCLS (2003)

	Susceptible (MIC µg/ml)	Intermediate susceptibility (MIC µg/ml)	Resistant (MIC µg/ml)
Penicillin	≤ 0.12	-	≥ 0.25
Amoxicillin	≤ 0.25	-	≥0.5
Erythromycin	≤ 0.5	1-4	≥ 8
Azithromycin	≤ 2	4	≥ 8
Clarithromycin	≤ 2	4	≥ 8
Clindamycin	≤ 0.5	1-2	≥ 4
Gentamicin	≤ 4	8	≥ 16
Cephalothin	≤ 8	16	≥ 32
Cephalexin	≤ 8	16	≥ 32
Vancomycin	≤ 4	8-16	≥ 32
Teicoplanin	≤ 8	16	≥ 32

RESULTS

Reproducibility

The type strain *Staph. aureus* NCTC 6571 was included in each run for the E test (n = 7) and vancomycin agar dilution (n = 4). The MIC values are shown in Table 56. The MIC values for the type strains of CNS for the 11 antibiotics tested are shown in Table 57.

Table 56: Reproducibility of Antibiotic Sensitivity Testing of *Staph. aureus* NCTC 6571

	Staph. aureus (n = 7)		
	MIC_{50}	MIC_{90}	Min-Max
Penicillin	0.032	0.064	0.023-0.064
Amoxicillin	0.047	0.094	0.032-0.094
Erythromycin	0.19	0.38	0.19-0.38
Azithromycin	0.75	1.0	0.75-1.0
Clarithromycin	0.19	0.25	0.19-0.25
Clindamycin	0.094	0.19	0.094-0.19
Gentamicin	0.5	0.75	0.25-0.75
Cephalexin	0.094	0.094	0.047-0.094
Cephalothin	0.75	1.0	0.75-1.0
Teicoplanin	1.5	3.0	1.0-3.0
Vancomycin	3.0	4.0	2.0-4.0
Van (AgD)*	1.0	1.0	1.0-2.0

*Vancomycin susceptibility testing using agar dilution technique

Table 57: MIC Values (μg/ml) for CNS Type Strains to the Antibiotics Tested

	Staph. epidermidis NCTC 11047	*Staph. hominis* NCTC 11320	*Staph. warneri* NCTC 10518	*Staph. capitis* NCTC 11045	*Staph. cohnii* NCTC 10515	*Staph. saprophyticus* NCTC 10516
Penicillin	0.047	0.032	0.032	0.047	0.25	0.25
Amoxicillin	0.094	0.094	0.125	0.064	0.25	0.25
Erythromycin	0.38	0.19	0.125	0.25	0.5	0.38
Azithromycin	0.75	0.5	0.38	0.75	0.75	0.75
Clarithromycin	0.38	0.38	0.25	0.38	0.25	0.25
Clindamycin	0.25	0.094	0.19	0.19	0.25	0.38
Gentamicin	0.047	0.032	0.047	0.064	0.016	0.023
Cephalexin	2	0.5	0.094	0.094	0.5	1
Cephalothin	0.125	0.125	2	1.5	4	6
Teicoplanin	1	1	1.5	0.75	1.5	4
Vancomycin	2	2	2	2	2	3
Van (AgD)*	2	1	2	1	1	2

*Vancomycin susceptibility testing using agar dilution technique

CNS Isolates from Blood Samples

The MIC frequency distribution, MIC_{50}, MIC_{90} and range of the 89 staphylococci for each antibiotic are shown in Table 58 andTable 59. The number and proportion of staphylococci susceptible, of intermediate susceptibility and resistant to each antibiotic are shown in Table 60. Vancomycin susceptibility using both the E test method and agar dilution are shown.

Penicillin

Seventeen isolates (19%) were susceptible to penicillin (MIC $\leq$ 0.12 μg/ml). Resistance to penicillin (MIC $\geq$ 0.25 μg/ml) was observed in 72 isolates (81%).

Amoxicillin

Twenty-two isolates (25%) were susceptible (MIC $\leq$ 0.25 μg/ml) and 67 isolates (75%) were resistant to amoxicillin (MIC $\geq$ 0.5 μg/ml).

Erythromycin

Twenty-eight isolates (32%) were susceptible to erythromycin (MIC $\leq$ 1 μg/ml), one was of intermediate susceptibility (MIC = 1-4 μg/ml) and 60 isolates (67%) were resistant (MIC $\geq$ 8 μg/ml).

Azithromycin

Twenty-nine isolates (33%) were susceptible to azithromycin (MIC $\leq$ 2 μg/ml) and 60 isolates (67%) were resistant (MIC $\geq$ 8 μg/ml).

Clarithromycin

Twenty-nine isolates (33%) were susceptible to clarithromycin (MIC $\leq$ 2.0 μg/ml) and 60 isolates (67%) were resistant (MIC $\geq$ 8 μg/ml).

Clindamycin

Seventy-two isolates (81%) were susceptible to clindamycin (MIC $\leq$ 0.5 μg/ml), two isolates (2%) were of intermediate susceptibility (MIC = 1-2 μg/ml) and 15 isolates (17%) were resistant (MIC $\geq$ 4 μg/ml).

Gentamicin

Eighty-seven isolates (98%) were susceptible to gentamicin (MIC $\leq$ 4 μg/ml), one isolate (1%) was of intermediate susceptibility (MIC = 8 μg/ml) and one isolate (1%) was resistant (MIC $\geq$ 16 μg/ml).

Cephalexin

Seventy isolates (79%) were susceptible to cephalexin (MIC $\leq$ 8 μg/ml), nine isolates (10%) were of intermediate susceptibility (MIC = 16 μg/ml) and ten isolates (11%) were resistant (MIC $\geq$ 32 μg/ml).

Cephalothin

Eighty-eight isolates (99%) were susceptible to cephalothin (MIC $\leq$ 8 μg/ml) and one isolate was of intermediate susceptibility (MIC = 16 μg/ml).

Teicoplanin

Eighty-five isolates (96%) were susceptible to teicoplanin (MIC $\leq$ 8 μg/ml), one isolate (1%) was of intermediate susceptibility (MIC = 16 μg/ml) and three isolates (3%) were resistant (MIC $\geq$ 32 μg/ml).

Vancomycin

Seventy-nine (89%) were susceptible to vancomycin (MIC $\leq$ 4 μg/ml) using the E test and ten isolates (11%) were of intermediate susceptibility (MIC = 8-16 μg/ml).

Vancomycin – agar dilution

Eighty-eight isolates (99%) were susceptible to vancomycin (MIC $\leq$ 4 μg/ml) using the agar dilution technique and one isolate (1%) was of intermediate susceptibility (MIC = 8-16 μg/ml).

Table 58: Frequency Distribution of Antimicrobial Sensitivity for CNS Isolated from Blood Samples

	Number of CNS clinical isolates for which the MIC (µg/ml) was:																									
	0.016	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**64**	**96**	**≥128**
PG	2	4	8	2	0	0	1	1	5	2	4	4	8	5	5	6	3	4	3	1	4	3	2	1	3	2	6
AC	2	1	2	1	8	2	1	-	5	2	10	3	6	8	7	2	6	1	2	2	3	3	3	2	2	-	5
EM	1	-	2	-	-	-	4	5	7	4	5	1	-	-	-	-	-	-	-	-	1	-	1	2	4	-	52
AZ	2	-	1	-	-	-	-	-	2	1	6	9	4	4	-	-	-	-	-	-	1	-	-	1	-	3	55
CH	2	-	1	-	-	-	5	4	4	6	6	1	-	-	-	-	-	-	-	1	-	-	3	2	1	3	50
CM	-	-	1	-	3	8	21	19	13	3	4	1	1	-	-	-	-	-	-	-	-	1	-	-	-	-	14
GM	4	11	26	13	19	2	5	1	1	3	-	1	1	-	-	-	-	1	-	-	1	-	-	-	-	-	-
CX	-	-	-	-	1	1	-	-	1	3	4	8	19	14	7	5	2	-	5	8	1	3	2	2	1		2
CE	-	-	2	4	8	6	12	12	15	6	14	5	2	1	1	-	-	-	-	1	-	-	-	-	-	-	-
TP	-	-	-	-	1	-	1	3	2	8	3	4	6	7	11	15	13	8	3	-	1	1	-	-	1	-	1
VA	-	-	-	-	-	-	-	-	1	2	-	3	6	17	28	22	3	6	1	-	-	-	-	-	-	-	-
V(AgD)*	-	-	-	-	-	-	-	-	-	-	1	-	38	-	35	-	14	-	1	-	-	-	-	-	-	-	-

*Vancomycin susceptibility testing using agar dilution technique
PG = penicillin, AC = amoxicillin, EM = erythromycin, AZ = azithromycin, CH = clarithromycin, CM = clindamycin, GM = gentamicin, CX = cephalexin, CE = cephalothin, TP = teicoplanin, VA = vancomycin

Table 59: MIC_{50} and MIC_{90} Values for CNS Clinical Isolates

(n = 89)	MIC_{50}	MIC_{90}	Range
Penicillin	1.5	64	≤0.016 – 256
Amoxicillin	1.5	48	≤0.016 – 256
Erythromycin	256	256	0.016 – 256
Azithromycin	256	256	0.016 – 256
Clarithromycin	256	256	0.016 – 256
Clindamycin	0.19	256	0.032 – 256
Gentamicin	0.047	0.23	0.016 – 16
Cephalexin	1.5	24	0.064 - 256
Cephalothin	0.25	0.75	0.032 – 12
Teicoplanin	2	6	0.064 – 256
Vancomycin	3	6	0.38 – 12
Vancomycin - (AgD)*	2	4	0.5 - 8

n = number of isolates
*Vancomycin susceptibility testing using agar dilution technique

Table 60: Susceptibility, Intermediate Susceptibility and Resistance of CNS Clinical Isolates

(n = 89)	Susceptible		Intermediate susceptibility		Resistant	
	n	%	n	%	n	%
Penicillin	17	19	-	-	72	81
Amoxicillin	22	25	-	-	67	75
Erythromycin	28	32	1	1	60	67
Azithromycin	29	33	-	-	60	67
Clarithromycin	29	33	-	-	60	67
Clindamycin	72	81	2	2	15	17
Gentamicin	87	98	1	1	1	1
Cephalexin	70	79	9	10	10	11
Cephalothin	88	99	1	1	-	-
Teicoplanin	85	96	1	1	3	3
Vancomycin	79	89	10	11	-	-
Vancomycin - (AgD)*	88	99	1	1	-	-

n = number of isolates
*Vancomycin susceptibility testing using agar dilution technique

Correlation Between the MIC's of Erythromycin and Clarithromycin and Azithromycin

There was a high correlation between the MIC's of erythromycin and azithromycin and erythromycin and clarithromycin for the CNS tested (Table 61).

Table 61: Correlation Between MIC's of Erythromycin versus Azithromycin and Clarithromycin

		Clarithromycin	Azithromycin
Erythromycin	Rho	0.97	0.99
	p-value	$p \leq 0.0001$	$p \leq 0.0001$

rho = Spearman's rank correlation

Correlation Between MIC's of Penicillin and the Antibiotics Tested

There was a significant correlation between the MIC's of penicillin and amoxicillin, cephalothin, cephalexin, erythromycin, clindamycin, teicoplanin and vancomycin. There was no correlation between MIC's of penicillin and those of gentamicin (Table 62).

Table 62: Correlation Between MIC's of Penicillin versus Antibiotics Tested

		Amoxicillin	Cephalothin	Cephalexin	Erythromycin	Gentamicin	Clindamycin	Teicoplanin	Vancomycin
Penicillin	Rho	0.097	0.77	0.73	0.28	0.014	0.37	0.34	0.25
	p value	$p \leq 0.0001$*	$p \leq 0.0001$*	$p \leq 0.0001$*	$p \leq 0.006$*	$p \leq 0.51$	$p \leq 0.0001$*	$p \leq 0.001$*	$p \leq 0.01$*

rho = Spearman's rank correlation
*Statistically significant

Antibiotic Sensitivity of Each *Staphylococcus* Species

Staph. epidermidis

The MIC's for *Staph. epidermidis* (n = 29) are shown in Table 63. Only 14% of the isolates were susceptible to penicillin and amoxicillin. Susceptibility to erythromycin, azithromycin and clarithromycin was higher, 41%. Twenty-five isolates (86%) were susceptible to clindamycin. All isolates were susceptible to gentamicin and cephalothin. Ninety-three percent were susceptible to cephalexin and teicoplanin. All isolates were susceptible to vancomycin using both the E test and the agar dilution technique.

Staph. hominis

The MIC's for *Staph. hominis* (n = 44) are shown in Table 63. Twenty-five percent of the isolates were susceptible to penicillin and amoxicillin. Susceptibility to erythromycin, azithromycin and clarithromycin was only seen in 7%, 5% and 7%, respectively. Thirty-three isolates (75%) were susceptible to clindamycin. All isolates were susceptible to gentamicin and cephalothin. Eighty-nine percent were susceptible to cephalexin. Susceptibility to teicoplanin was seen in 98% of isolates. All isolates were susceptible to vancomycin using both the E test and the agar dilution technique.

Staph. warneri

The MIC's for *Staph. warneri* (n = 9) are shown in Table 63. Twenty-two percent of the isolates were susceptible to penicillin and amoxicillin. Susceptibility to erythromycin, azithromycin and clarithromycin was seen in 56% of the isolates. All isolates were susceptible to clindamycin, cephalothin and teicoplanin. Eighty-nine percent were susceptible to gentamicin. Seventy-eight percent of isolates were susceptible to cephalexin. Susceptibility to teicoplanin was seen in 98% of isolates. All isolates were susceptible to vancomycin using both the E test and the agar dilution technique.

Table 63: Range, MIC_{50} and MIC_{90} for Each *Staphylococcus* sp. and Proportion Susceptible to the Antibiotics Tested

Species	Antibiotic	MIC (µg/ml)			S% (S and I %)
		Range	50	90	
Staph. epidermidis (n = 29)	Penicillin	0.016-48	1	24	14
	Amoxicillin	0.016-64	1	16	14
	Erythromycin	0.125-256	64	256	41
	Azithromycin	0.5-256	96	256	41
	Clarithromycin	0.125-256	96	256	41
	Clindamycin	0.064-256	0.19	256	83 (86)
	Gentamicin	0.023-0.38	0.032	0.125	100
	Cephalexin	0.094-32	2	15	83 (93)
	Cephalothin	0.032-1	0.19	0.5	100
	Teicoplanin	0.25-64	3	16	90 (93)
	Vancomycin	0.38-12	3	8	79 (100)
	Van (AgD)*	0.5-8	2	8	97 (100)
Staph. hominis (n = 44)	Penicillin	0.016-256	2	96	25
	Amoxicillin	0.016-256	1.5	48	25
	Erythromycin	0.016-256	256	256	7
	Azithromycin	0.016-256	256	256	5
	Clarithromycin	0.016-256	256	256	7
	Clindamycin	0.032-256	0.19	256	75
	Gentamicin	0.016-1	0.047	0.064	100
	Cephalexin	0.064-48	1.5	24	82 (89)
	Cephalothin	0.032-1	0.19	0.5	100
	Teicoplanin	0.064-256	1.5	4	98
	Vancomycin	0.5-8	3	4	91 (100)
	Van (AgD)*	1-4	1	2	100
Staph. warneri (n = 9)	Penicillin	0.25-256	8	96	22
	Amoxicillin	0.125-256	4	256	22
	Erythromycin	0.032-256	0.19	256	56
	Azithromycin	0.016-256	1	256	56
	Clarithromycin	0.016-256	0.25	256	56
	Clindamycin	0.094-0.5	0.125	0.5	100
	Gentamicin	0.032-16	0.25	16	78 (89)
	Cephalexin	0.38-256	12	256	44 (78)
	Cephalothin	0.125-2	0.5	2	100
	Teicoplanin	0.125-8	1.5	8	100
	Vancomycin	1-6	2	6	89 (100)
	Van (AgD)*	1-4	1	2	100

*Vancomycin susceptibility testing using agar dilution technique
S = susceptible; I = intermediate susceptibility (MIC = µg/ml)

Staph. capitis and Staph. cohnii

The MIC's for individual isolates of *Staph. capitis* (n = 3) and *Staph. cohnii* (n = 3) are shown in Table 64.

Table 64: MIC Values and Susceptibility of *Staph. capitis* and *Staph. cohnii* to the Antibiotics Tested

	Staph. capitis (n = 3)		*Staph. cohnii* (n = 3)	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Penicillin	0.023	S	0.094	S
	0.023	S	0.125	S
	1	R	2	R
Amoxicillin	0.032	S	0.25	S
	0.032	S	0.25	S
	2	R	2	R
Erythromycin	0.25	S	32	R
	0.38	S	48	R
	0.75	I	256	R
Azithromycin	0.75	S	48	R
	1	S	96	R
	1.5	S	256	R
Clarithromycin	0.25	S	32	R
	0.38	S	32	R
	1	S	256	R
Clindamycin	0.19	S	0.032	S
	0.19	S	0.047	S
	0.25	S	0.047	S
Gentamicin	0.016	S	0.25	S
	0.064	S	0.5	S
	0.125	S	1	S
Cephalexin	0.25	S	1	S
	0.75	S	3	S
	1	S	256	R
Cephalothin	0.047	S	0.38	S
	0.094	S	0.5	S
	0.125	S	12	I
Teicoplanin	0.19	S	4	S
	0.38	S	4	S
	6	S	6	S
Vancomycin	1	S	3	S
	3	S	3	S
	4	S	4	S
Van (AgD)*	1	S	1	S
	2	S	2	S
	2	S	2	S

*Vancomycin susceptibility testing using agar dilution technique
S = susceptible; I = intermediate susceptibility; R = resistant
(MIC = µg/ml)

Staph. saprophyticus

The MIC's for *Staph. saprophyticus* (n = 1) are shown in Table 65.

Table 65: MIC Values and Susceptibility of *Staph. saprophyticus* to the Antibiotics Tested

	Staph. saprophyticus (n = 1)	
	MIC	**Susceptibility**
Penicillin	0.5	R
Amoxicillin	0.5	R
Erythromycin	48	R
Azithromycin	192	R
Clarithromycin	48	R
Clindamycin	0.125	S
Gentamicin	0.064	S
Cephalexin	2	S
Cephalothin	0.38	S
Teicoplanin	3	S
Vancomycin	3	S
Van (AgD)*	2	S

*Vancomycin susceptibility testing using agar dilution technique
S = susceptible; I = intermediate susceptibility; R = resistant
(MIC = µg/ml)

II. Antibiotic Sensitivity of Other Bacteria

Materials and Methods

Antibiotic sensitivity testing was carried out for the following bacteria: *Rothia* spp., *Actinomyces* spp., *Propionibacterium acnes*, *Micrococcus luteus*, *Neisseria* spp., *Haemophilus parainfluenzae*, *Aerococcus viridans* and *Abiotrophia defectiva*. This was determined using E test strips (AB Biodisk) for the same antibiotics described in Chapter 8.

Type Strains

The following type strains were included: *Actinomyces naeslundii* NCTC 10301, *Propionibacterium acnes* NCTC 737, *Rothia dentocariosa* NCTC 10207, *Micrococcus luteus* NCTC 2665, *Neisseria mucosa* NCTC 10775, *Haemophilus influenzae* NCTC 11931 and *Aerococcus viridans* NCTC 7592. The type strain *Staph. aureus* NCTC 6571 was included in each run for the E test (n = 5).

E test Method

Direct colony suspension was used to prepare the inoculum for antibiotic sensitivity testing using the E test as described in Chapter 8. MHA supplemented with 5% defibrinated horse blood in a 150 mm plate at a depth of 4.0 ± 0.5 mm was used in the same way as described earlier. For *Actinomyces* and *Propionibacterium* spp. the plates were incubated for 5–7 days anaerobically at 37°C. For the remaining bacteria, the plates were incubated for 20-24 hours in 5% CO_2. The MIC value for each antibiotic was recorded as the lowest point where the edge of the elliptical zone of inhibition intersected the MIC scale on the strip.

Interpretation of Susceptibility Categories

Propionibacterium acnes isolates were classified as being susceptible, of intermediate susceptibility or resistant according to the interpretive standards for anaerobic bacteria (NCCLS 2001) shown in Table 66. MIC interpretive standards are not available for erythromycin, azithromycin, clarithromycin, gentamicin, teicoplanin and vancomycin and susceptibility categorization for these antibiotics could not be performed.

Table 66: Breakpoints of Antibiotics for Anaerobic Bacteria According to the Interpretive Standards of the NCCLS (2001)

	Susceptible (MIC µg/ml)	Intermediate susceptibility (MIC µg/ml)	Resistant (MIC µg/ml)
Penicillin	≤0.5	1	≥2
Amoxicillin	≤0.5	1	≥2
Clindamycin	≤2	4	≥8
Cephalosporins	≤16	32	≥64

Interpretive standards for *Haemophilus influenzae* (NCCLS 2003) were used for *Haemophilus parainfluenzae* susceptibility categorization (Table 67). Ampicillin breakpoints were used for amoxicillin as recommended by the NCCLS (2003). MIC interpretive standards were not available for penicillin, erythromycin, gentamicin, cephalexin, cephazolin, clindamycin, teicoplanin and vancomycin and susceptibility categorization for these antibiotics could not be performed.

Table 67: Breakpoints of Antibiotics for *Haemophilus influenzae* According to the Interpretive Standards of the NCCLS (2003)

	Susceptible (MIC µg/ml)	Intermediate susceptibility (MIC µg/ml)	Resistant (MIC µg/ml)
Amoxicillin	≤1	2	≥4
Azithromycin	≤4	-	-
Clarithromycin	≤8	16	≥32

Interpretive standards for *Neisseria gonorrhoeae* (NCCLS 2003) to penicillin were used for susceptibility categorization of *Neisseria* spp. (Table 68). Interpretive standards for the remainder of the antibiotics tested were not available.

Table 68: Breakpoints of Penicillin for *Neisseria gonorrhoeae* According to the Interpretive Standards of the NCCLS (2001)

	Susceptible (MIC µg/ml)	Intermediate susceptibility (MIC µg/ml)	Resistant (MIC µg/ml)
Penicillin	≤0.06	0.12-1	≥2

MIC interpretive standards for *Staphylococcus* spp. (Table 55 page 185) were used for susceptibility categorization of *Micrococcus luteus*. MIC interpretive standards for *Streptococcus* spp. (Table 43 page 164) were used for susceptibility categorization of *Aerococcus viridans* and *Abiotrophia defectiva*. There are no MIC interpretive standards available for *Rothia* and *Actinomyces* spp. and susceptibility categorization for these bacteria could not be performed.

RESULTS

Reproducibility

The type strain *Staph. aureus* NCTC 6571 was included in each run for the E test (n = 5) and the MIC_{50}, MIC_{90} and range of MIC's are shown in Table 69. The MIC values for the type strains of oral bacteria for the 11 antibiotics tested are shown in Table 70.

Table 69: Reproducibility of Antibiotic Sensitivity Testing of *Staph. aureus* NCTC 6571

	Staph. aureus (n = 5)		
	MIC_{50}	MIC_{90}	Min-Max
Penicillin	0.032	0.064	0.023-0.064
Amoxicillin	0.047	0.094	0.032-0.094
Erythromycin	0.19	0.38	0.19-0.38
Azithromycin	0.75	1.0	0.75-1.0
Clarithromycin	0.19	0.25	0.19-0.25
Clindamycin	0.094	0.19	0.094-0.19
Gentamicin	0.5	0.75	0.25-0.75
Cephalexin	0.094	0.094	0.047-0.094
Cephalothin	0.75	1.0	0.75-1.0
Teicoplanin	1.5	3.0	1.0-3.0
Vancomycin	3.0	4.0	2.0-4.0

Clinical Isolates

Rothia spp.

The MIC_{50}, MIC_{90} and range for *Rothia* spp. (n = 11) for each antibiotic are shown in Table 71. MIC interpretive standards for *Rothia* spp. are not provided by the NCCLS and susceptibility categorization could not be performed for them.

Actinomyces spp.

The MIC_{50}, MIC_{90} and range for *Actinomyces* spp. (n = 6) for each antibiotic are shown in Table 72. MIC interpretive standards for *Actinomyces* spp. are not provided by the NCCLS and susceptibility categorization could not be performed for them.

Table 70: MIC Values (µg/ml) for Bacterial Type Strains to the Antibiotics Tested

	Actinomyces naeslundii NCTC 10301	*Propionibacterium acnes* NCTC 737	*Rothia dentocariosa* NCTC 10207	*Micrococcus luteus* NCTC 2665	*Neisseria mucosa* NCTC 10774	*Haemophilus influenzae* NCTC 11931	*Aerococcus viridans* NCTC 7592
Penicillin	0.016	0.016	0.016	0.094	0.047	0.016	0.125
Amoxicillin	0.016	0.016	0.023	0.047	0.032	0.016	0.25
Erythromycin	0.023	0.016	0.032	0.19	0.064	0.016	0.38
Azithromycin	0.047	0.016	0.064	0.25	0.094	0.016	0.5
Clarithromycin	0.023	0.016	0.023	0.19	0.064	0.016	0.19
Clindamycin	0.047	0.38	0.25	0.125	0.047	0.5	0.032
Gentamicin	0.023	0.016	6	0.094	0.032	0.016	4
Cephalexin	1.5	0.016	0.19	0.5	0.125	12	0.25
Cephalothin	0.023	0.016	0.023	0.38	4	0.75	0.5
Teicoplanin	0.064	0.19	0.75	0.094	0.38	64	0.094
Vancomycin	0.5	0.38	2	0.38	1.5	2	0.38

Table 71: MIC_{50} and MIC_{90} Values for *Rothia* spp.

(n =11)	MIC_{50}	MIC_{90}	Range
Penicillin	0.064	0.19	0.016-0.75
Amoxicillin	0.032	0.125	0.016-1
Erythromycin	0.016	0.125	0.016-1.5
Azithromycin	0.047	1.5	0.016-2
Clarithromycin	0.016	0.125	0.016-1
Clindamycin	2	4	0.125-12
Gentamicin	0.125	2	0.032-2
Cephalexin	1	3	0.032-48
Cephalothin	0.094	0.25	0.016-6
Teicoplanin	0.25	0.5	0.032-0.75
Vancomycin	1.5	2	0.5-2

n = number of isolates
Rothia mucilaginosa (n = 6); *Rothia dentocariosa* (n = 5)

Table 72: MIC_{50} and MIC_{90} Values for *Actinomyces* spp.

(n = 6)	MIC_{50}	MIC_{90}	Range
Penicillin	0.016	0.25	0.016-0.25
Amoxicillin	0.016	0.38	0.016-0.38
Erythromycin	0.016	0.023	0.016-0.047
Azithromycin	0.016	0.094	0.016-0.094
Clarithromycin	0.016	0.023	0.016-0.032
Clindamycin	0.19	2	0.016-2
Gentamicin	0.19	6	0.016-6
Cephalexin	0.19	3	0.064-3
Cephalothin	0.094	0.38	0.064-0.38
Teicoplanin	0.19	2	0.094-2
Vancomycin	0.75	2	0.5-2

n = number of isolates
A. naeslundii (n = 3), *A. viscous* (n = 2), *A. odontolyticus* (n = 1)

Propionibacterium acnes

The MIC_{50}, MIC_{90} and range for *P. acnes* isolates (n = 8) for each antibiotic are shown in Table 73. The number and proportion susceptible, of intermediate susceptibility and resistant to penicillin, amoxicillin, clindamycin and cephalosporins are shown in Table 74. Susceptibility breakpoints for erythromycin, azithromycin, clarithromycin, gentamicin, teicoplanin and vancomycin are not provided by the NCCLS and susceptibility categorization could not be carried out for them.

Table 73: MIC_{50} and MIC_{90} Values for *P. acnes*

(n = 8)	MIC_{50}	MIC_{90}	Range
Penicillin	0.016	0.016	0.016
Amoxicillin	0.016	0.016	0.016
Erythromycin	0.016	0.016	0.016
Azithromycin	0.016	0.016	0.016
Clarithromycin	0.016	0.016	0.016
Clindamycin	0.016	0.094	0.016-0.094
Gentamicin	0.023	0.5	0.016-0.5
Cephalexin	0.047	0.19	0.032-0.19
Cephalothin	0.023	0.064	0.016-0.064
Teicoplanin	0.19	0.5	0.094-0.5
Vancomycin	0.5	1	0.38-1

n = number of isolates

Table 74: Susceptibility, Intermediate Susceptibility and Resistance of *P. acnes*

(n = 8)	Susceptible		Intermediate susceptibility		Resistant	
	n	%	n	%	n	%
Penicillin	8	100	-	-	-	-
Amoxicillin	8	100	-	-	-	-
Clindamycin	8	100	-	-	-	-
Cephalexin	8	100	-	-	-	-
Cephalothin	8	100	-	-	-	-

n = number of isolates

Micrococcus luteus

The MIC_{50}, MIC_{90} and range for *Micrococcus luteus* isolates (n = 5) for each antibiotic are shown in Table 75. The number and proportion susceptible, of intermediate susceptibility and resistant to each antibiotic are shown in Table 76. MIC interpretive standards for *Staphylococcus* spp. were used (NCCLS 2003).

Table 75: MIC_{50} and MIC_{90} Values for *M. luteus*

(n = 5)	MIC_{50}	MIC_{90}	Range
Penicillin	0.125	0.19	0.094-0.19
Amoxicillin	0.125	0.25	0.064-0.25
Erythromycin	0.125	32	0.094-32
Azithromycin	0.125	12	0.064-12
Clarithromycin	0.125	12	0.094-12
Clindamycin	0.125	12	0.125-0.25
Gentamicin	0.38	0.75	0.19-0.75
Cephalexin	0.75	1	0.38-1
Cephalothin	0.25	0.38	0.19-0.38
Teicoplanin	0.25	0.38	0.19-0.38
Vancomycin	0.5	1	0.25-1

Table 76: Susceptibility, Intermediate Susceptibility and Resistance of *M. luteus*

(n = 5)	Susceptible		Intermediate susceptibility		Resistant	
	n	%	n	%	n	%
Penicillin	3	60	-	-	2	40
Amoxicillin	5	100	-	-	-	-
Erythromycin	4	80	-	-	1	20
Azithromycin	4	80	-	-	1	20
Clarithromycin	4	80	-	-	1	20
Clindamycin	5	100	-	-	-	-
Cephalexin	5	100	-	-	-	-
Cephalothin	5	100	-	-	-	-
Teicoplanin	5	100	-	-	-	-
Vancomycin	5	100	-	-	-	-

***Neisseria* spp.**

The MIC values for *Neisseria* spp. (n = 2) and their susceptibility to penicillin are shown in Table 77. MIC interpretive standards are not available for the remainder of the antibiotics tested.

Haemophilus parainfluenzae

The MIC values for *H. parainfluenzae* (n = 2) and their susceptibility to amoxicillin, azithromycin and clarithromycin are shown in Table 77. MIC interpretive standards are not available for the remainder of the antibiotics tested.

Table 77: MIC Values and Susceptibility of *Neisseria* spp. and *H. parainfluenza*

	***Neisseria* spp.** (n = 2)		***Haemophilus* spp.** (n = 2)	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Penicillin	0.023	S	0.032	NA
	1	I	0.047	NA
Amoxicillin	0.064	NA	0.032	S
	0.75	NA	0.032	S
Erythromycin	0.032	NA	0.125	NA
	4	NA	0.5	NA
Azithromycin	0.064	NA	0.75	S
	1.5	NA	0.5	S
Clarithromycin	0.023	NA	1	S
	3	NA	0.75	S
Clindamycin	0.016	NA	0.125	NA
	4	NA	0.5	NA
Gentamicin	0.125	NA	6	NA
	8	NA	4	NA
Cephalexin	1.5	NA	2	NA
	24	NA	3	NA
Cephalothin	0.19	NA	0.19	NA
	4	NA	0.38	NA
Teicoplanin	0.19	NA	0.125	NA
	16	NA	0.25	NA
Vancomycin	0.75	NA	2	NA
	4	NA	1	NA

S = susceptible; I = intermediate susceptibility, R = resistant; (MIC = µg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS

Aerococcus viridans

The MIC values for *Aerococcus viridans* (n = 1) and its susceptibility to each antibiotic tested are shown in Table 78. MIC interpretive standards for *Streptococcus* spp. were used (NCCLS 2003).

Abiotrophia defectiva

The MIC values for *Abiotrophia defectiva* (n = 1) and its susceptibility to each antibiotic tested are shown in Table 78. MIC interpretive standards for *Streptococcus* spp. were used (NCCLS 2003).

Table 78: MIC Values and Susceptibility of *Aerococcus viridans* and *Abiotrophia defectiva*

	Aerococcus viridans (n = 1)		*Abiotrophia defectiva* (n = 1)	
	MIC	**Susceptibility**	**MIC**	**Susceptibility**
Penicillin	0.125	S	0.032	S
Amoxicillin	0.19	S	0.094	S
Erythromycin	0.19	S	0.094	S
Azithromycin	0.25	S	0.125	S
Clarithromycin	0.19	S	0.064	S
Clindamycin	0.064	S	0.094	S
Gentamicin	4	NA	3	NA
Cephalexin	0.5	S	0.25	S
Cephalothin	0.25	S	0.19	S
Teicoplanin	0.38	NA	0.5	S
Vancomycin	0.5	S	1	S

S = susceptible; I = intermediate susceptibility, R = resistant; (MIC = µg/ml)
NA = not applicable as no susceptibility categories provided by NCCLS

DISCUSSION

Multiple antibiotic resistance of CNS isolates has been reported (Pfaller & Herwaldt 1988; Sanzén & Walder 1988; Henwood et al 2000; Johnson et al 2003; Agvald-Öhman et al 2004). The majority of CNS isolated from intensive care patients in Sweden were found to be resistant to multiple antibiotics (Agvald-Öhman et al 2004). The rates of resistance were 95% for penicillin, 86% for oxacillin, 48% for erythromycin, 42% for clindamycin, 54% for gentamicin and 66% for ciprofloxacin. The prevalence of antibiotic-resistant CNS amongst 25 hospitals in the UK was determined over an 8 month period in 1999. Penicillin resistance was evident in 82% of CNS isolates and erythromycin resistance was present in 49% (Henwood et al 2000). Eighty percent of CNS isolated from blood cultures in France were resistant to penicillin and 43% were resistant to erythromycin (Decousser et al 2003). Eighty-four percent of CNS isolates from hospital patients in Kuwait were resistant to penicillin. Resistance to erythromycin was also present in 52% (Udo et al 1995).

These findings are in agreement with the results of the present work, where resistance to penicillin (MIC $\geq$ 0.25 μg/ml) was observed in 72 CNS isolates (81%) and erythromycin resistance (MIC $\geq$ 8 μg/ml) was seen in 60 isolates (67%). In a recent study, 70% of oral staphylococci isolated from the subgingival crevice of periodontitis patients were found to be resistant to penicillin (Murdoch et al 2004).

Methicillin resistance has been found to predict resistance to multiple classes of antibiotics in addition to β-lactams (Johnson et al 2003; Agvald-Öhman et al 2004). Three quarters of methicillin-resistant CNS and one quarter to one third of methicillin susceptible CNS from clinical specimens were reported to be resistant to erythromycin (Archer & Climo 1994). An analysis of 177 methicillin-resistant CNS from different geographic areas found 50% to be resistant to trimethoprim, 75% to be resistant to erythromycin, 60% to be resistant to clindamycin and 35% resistant to tetracycline (Archer & Scott 1990). In the present study, of those CNS isolates resistant to penicillin, 65% were also resistant to erythromycin and 19% were resistant to clindamycin. There was a significant correlation between the MIC's of penicillin and those of amoxicillin, erythromycin, clindamycin, cephalothin, cephalexin, teicoplanin and vancomycin.

Macrolides and cephalosporins have been suggested as alternatives for IE prophylaxis. The efficacy of macrolide antibiotics are markedly compromised in practice by widespread resistance to their action. Erythromycin resistance was observed in 67% of CNS isolates in the present work. Among the new macrolides, clarithromycin and azithromycin offer no advantage over erythromycin for CNS. They have equal activities *in vitro* against susceptible isolates and

there is cross resistance among resistant isolates (Maskell et al 1990). This is further substantiated by the results of the present study where a significant correlation was found between the MIC's of erythromycin and those of azithromycin and clarithromycin.

None of the CNS tested were resistant to cephalothin but 11% were resistant to cephalexin. Researchers investigating the antibiotic susceptibility of CNS found different levels of resistance to three different cephalosporins. Ten percent were resistant to cefepime, 39% to ceftazidime and 12% to ceftriaxone (Fritsche et al 2003).

While virtually all CNS, with the exception of *Staph. haemolyticus,* remain susceptible to vancomycin, isolates have become less susceptible to teicoplanin (Goldstein et al 1990; Bannerman et al 1991; Cercenado et al 1996; Lallemand et al 2002; Maugein et al 2004). This is especially true for CNS isolated from patients in Europe where teicoplanin is approved for general use. In two studies in France, teicoplanin MIC's were 8 to 16 μg/ml for 23% and 38% of CNS and 32 μg/ml for 1.7% and 3.2%, indicating resistance (Goldstein et al 1990; Maugein et al 2004). Virtually all of the isolates in the previous studies were methicillin-resistant. None of the isolates showed reduced susceptibility to vancomycin but in one study teicoplanin resistance was correlated with either vancomycin or teicoplanin use (Maugein et al 2004). This is in agreement with the results of the present work where 3% of CNS isolates were resistant to teicoplanin and one was of intermediate susceptibility. None of the isolates in the study reported here were resistant to vancomycin.

Vancomycin agar dilution suspectibility testing was performed for all CNS isolates in addition to the E test. The E test showed slightly higher MIC's than agar dilution. Only one isolate had intermediate susceptibility using agar dilution compared to 10 isolates using the E test. The remainder of the isolates were all susceptible to vancomycin. Similar results were reported by a group of workers investigating CNS antibiotic susceptibility using agar dilution. They found only two isolates out of 239 with intermediate susceptibility to vancomycin and the remainder were fully susceptible (Del' Alamo et al 1999).

Differences have been recognized in the susceptibility patterns of different species of CNS. Analysis of *Staph. epidermidis* isolates causing prosthetic valve endocarditis within one year following surgery found 84% to be resistant to methicillin (Karchmer et al 1983). Thirteen out of 19 *Staph. epidermidis* strains isolated from another group of patients with IE were resistant to multiple antibiotics (Etienne et al 1988). Eighty-one percent of *Staph. epidermidis* isolates from patients in a Kuwait hospital were resistant to three or more antibiotics tested (Udo et al 1995). *Staph. epidermidis* isolated from urine showed high levels of resistance to penicillin, ampicillin,

erythromycin, clindamycin and gentamicin (Bourgault & Gauvreau 1983). This is similar to the results of this study where 86% of *Staph. epidermidis* isolates were resistant to penicillin and amoxicillin and 59% were resistant to erythromycin. Reduced susceptibility to teicoplanin in this study was most commonly seen in *Staph. epidermidis* and *Staph. hominis*. Similarly, other workers in the United States found that *Staph. epidermidis* was the most common isolate to show reduced susceptibility to teicoplanin (Bannerman et al 1991). This is particularly significant as *Staph. epidermidis* comprised 36% of CNS isolated following dento-gingival manipulative procedures in this study and accounts for the majority of IE caused by CNS (Richardson et al 1984; Hoen et al 2002).

Staph. hominis and *Staph. warneri* showed similar patterns of resistance to *Staph. epidermidis* (Bourgault & Gauvreau 1983). In the present work, 75% of *Staph. hominis* and 78% of *Staph. warneri* were resistant to penicillin. Ninety-three percent of *Staph. hominis* isolates and 44% of *Staph. warneri* were resistant to erythromycin.

Methicillin resistance is uncommon among *Staph. capitis* isolates but it has been reported in cases of IE (Banders & Dariouiche 1992; Terada et al 1996). In the present work, only one of the three *Staph. capitis* isolates was resistant to penicillin, but they were susceptible to the remainder of the antibiotics tested. All three isolates of *Staph. cohnii* were resistant to erythromycin while one isolate was resistant to penicillin and cephalexin. The single *Staph. saprophyticus* isolate was resistant to both erythromycin and penicillin.

A recent increase in the number of cases of *Rothia* spp. as pathogens has been reported (Ascher et al 1991; Hopkins et al 1992; McWhinney et al 1992; Binder et al 1997; Llopis & Carratala 2000; Salamon & Prag 2002) with one report of IE caused by *Rothia mucilaginosa* which was relatively resistant to penicillin and cephalothin (Pinsky et al 1989). The results of antibiotic susceptibility testing of *Rothia* spp. in the present work revealed low MIC_{50} and MIC_{90} values for penicillin, amoxicillin, the cephalosporins and the macrolides. Gentamicin and vancomycin MIC's were on the borderline and definite susceptibility could not be verified due to the absence of interpretive standards by the NCCLS. These results are in agreement with earlier work showing the same pattern of susceptibility for *Rothia dentocariosa* isolated from patients with bacteraemia in Denmark (Salamon & Prag 2002). Other workers have found *Rothia* spp. implicated in IE to be susceptible to a range of antibiotics including ampicillin, cephalothin, chloramphenicol, gentamicin, oxacillin, penicillin, clindamycin erythromycin, vancomycin and tetracycline (Rubin et al 1978; Prag et al 1985; Coudron et al 1987).

Actinomyces spp. in the present study showed low MIC values to most of the antibiotics tested. Penicillin and other β-lactams have been found to be the most active agents of those tested against *Actinomyces* species (Goldstein et al 2003). *A. odontolyticus* isolated from an abscess was susceptible to amoxicillin/clavulanate, cefoxitin, clindamycin and metronidazole, but resistant to ciprofloxacin (Sofianou et al 2004).

Propionibacterium spp. are associated with serious infections (Lewis & Abramson 1980; Mathisen et al 1984; Brook & Frazier 1993; Günthard et al 1994) and have been isolated from blood following dental extractions (Okabe et al 1995; Roberts et al 1998b) and endodontic treatment (Debelian et al 1995). In the present work, *P. acnes* was susceptible to all of the antibiotics tested. This is in agreement with other researchers (Günthard et al 1994; Shore et al 1999; Goldstein et al 2003)

Nine percent of *Neisseria* spp. isolated from plaque samples from healthy children were resistant to ampicillin and 23% were resistant to erythromycin (Ready et al 2003). Of the two isolates tested for antibiotic sensitivity in this study, one was susceptible to penicillin and the other showed intermediate susceptibility. This second isolate also had elevated MIC values for erythromycin, azithromycin, clarithromycin, gentamicin, cephalexin, clindamycin, teicoplanin and vancomycin. Whether this signifies resistance is not definite as interpretive standards for these antibiotics are not provided by the NCCLS.

Haemophilus parainfluenzae isolates showed low MIC values for most of the antibiotics tested in this study. This is in agreement with other workers who reported that *Haemophilus influenzae* isolated from patients in intensive care units in Europe were completely susceptible to most of the β-lactams tested (Fluit et al 2001). Conversely, ampicillin resistance was evident in 39% of *Haemophilus influenzae* isolated from medical centres in Taiwan (Chang et al 2000). This was attributed to the excessive use and over the counter availability of antibiotics in the country.

Summary

A large percentage of CNS isolates were found to be resistant to penicillin and macrolide antibiotics. These antibiotics are the main groups used for prophylaxis of IE. A similar pattern of resistance was found in *M. luteus* isolates in the present study. *Rothia* spp., *Actinomyces* spp., *Propionibacterium acnes*, *Haemophilus* spp., *Aerococcus viridans* and *Abiotrophia defectiva* were relatively susceptible to the antibiotics used for IE prophylaxis. One *Neisseria* sp. isolate had elevated MIC values for the macrolides, clindamycin, teicoplanin and

vancomycin and showed intermediate resistance to penicillin. This may be evidence of antibiotic resistance gene transfer between oral bacteria.

Resistance of oral microorganisms to common antibiotics may impair not only the prevention of IE but also the management of acute orofacial infections. It is therefore important to reassess the routine use of penicillin, the macrolides and cephalexin for antibiotic prophylaxis in at risk patients. This is especially important since azithromycin suspension has recently replaced clindamycin suspension for children in the UK (Littler 2001). It has also been recommended as an alternative to penicillin along with clindamycin for prophylaxis of IE by the American Heart Association (Dajani et al 1997).

CHAPTER 10
FINAL DISCUSSION

The general hypothesis that has been tested in this study is that conservative dental procedures cause a significant bacteraemia. The procedures investigated were placement of rubber dam, use of the fast and slow drill, placement of matrix band and wedge and placement of gingival retraction cord.

In the context of IE prophylaxis the term 'significant bacteraemia' requires elaboration. In clinical terms, a significant bacteraemia is one that will lead directly to the development of IE. Although this seems logical, it is complicated by the fact that it is impossible to confidently identify such a bacteraemia. For this reason, the use of statistical inference is the appropriate approach. This has been used recently in the Endocarditis Recommendations published by the British Cardiac Society and the Royal College of Surgeons of England, Faculty of Dental Surgery, the dental aspects of which have been published on the Royal College of Surgeons website (Roberts et al 2004). A significant bacteraemia was defined as a post-procedure bacteraemia that was significantly greater than baseline (pre-procedure). Therefore, it is essential to take before and after blood samples to enable interpretation of the data to determine whether or not there is a statistically significant difference. The difficulty of considering statistical significance as equivalent to clinical significance is not underestimated.

It is also important to consider the total duration of bacteraemia resulting from the dental manipulation. The total duration comprises two components. Firstly, the time taken for the bacteraemia to disappear once the dental procedure has stopped (Lucas et al 2001) and secondly the duration of the procedure (Delahaye & Gevigney 2001). Conservative dental procedures take a longer time and are likely to pose a greater risk of causing IE than single or multiple extractions which take only a few minutes. This may be because the intensity of bacteraemia from matrix band and wedge placement, for example, which is just one of the several procedures carried out for conservation, is similar to that following a single extraction. In terms of total exposure to infective microorganisms, conservative dental procedures pose a clinical risk as they are carried out more frequently than extractions (Al Karaawi et al 2001). This is one possible answer as to why antibiotic prophylaxis has not had any impact on the prevalence of IE (Van der Meer et al 1992).

A further difficulty is the emerging theory that 'everyday' bacteraemia from toothbrushing and mastication cause small but frequent bacteraemia cumulatively. This everyday bacteraemia over a set period such as one year (Al Karaawi et al 2001) represents a far greater risk than a

small and short duration bacteraemia from a single extraction. This has been the general theory of IE causation by low intensity but frequent bacteraemia (Drangsholt 1998).

Dental extractions and scaling have been classically considered to be the cause of IE. However, preliminary studies indicate that patients aware of an impending visit to the dentist make an extraordinary effort to clean their teeth causing a large bacteraemia before the dental treatment is performed. It is possible that this pre-treatment appointment toothbrushing is the cause of the bacteraemia that leads to seeding of the NBTV. This has emerged as the special theory of causation of IE (Lucas, personal communication). Antibiotic prophylaxis given for dental treatment is effective within 2 hours of the bacteraemia and therefore will not be effective against the bacteraemia caused by vigorous toothbrushing before leaving home.

The conventional categories of at risk patients are given by the Endocarditis Working Party for Antimicrobial Chemotherapy (Simmons 1993), the American Heart Association (Dajani et al 1997), European Cardiac Society (Horstkotte et al 2004) guidelines and the most recent recommendations from the British Cardiac Society and the Royal College of Surgeons of England (Roberts et al 2004). There have been recent developments with the American Heart Association from a closed meeting and the British Society of Antimicrobial Chemotherapy Guidelines Working Party in 2004. It is likely that the guidelines will show a reduced number of cardiac conditions for which antibiotic prophylaxis should be given. These include an earlier episode of IE, prosthetic valves, unrepaired ventricular septal defect and mitral valve prolapse with regurgitation and complex congenital heart disease. The new recommendations are at a relatively early stage of development and it is possible that they will change. The overall effect is likely to be a large reduction in the number of individuals requiring antibiotic prophylaxis. It is believed that the increased use of antibiotics prompted by the additional procedures recommended for antibiotic prophylaxis is offset by the smaller number of individuals considered at risk of developing IE.

The knowledge gained from the present work depends greatly on the use of the technique of lysis filtration. Although lysis filtration has been used in the dental context since 1977 (Hockett et al 1977) it was not until 2002 that a robust and scientifically based validation of this technique was published (Lucas et al 2002a). It was demonstrated that 100 µl aliquots of saline seeded with approximately 100 cfu/ml of a streptococcal type strain inoculated onto BHI agar, yielded very close counts to the number of cfu's estimated from the lysis filtration filters through which blood seeded with identical 100 µl aliquots was drawn. In other words, the cfu's that grew on the filters were the cfu's that had been inoculated. The work presented in this

study is based on this technique and the results are reliable estimates of the nature and quantity of microorganisms detected from blood samples following conservative dental procedures.

It is important to relate the current work with other studies looking at similar dental procedures. These were originally investigated using the broth culture technique (Roberts et al 2000). Although the prevalence was less with broth culture, the results of the present work using lysis filtration identified the same procedures as causing a statistically significant difference between baseline and post-procedure samples. Other workers have reported a significant bacteraemia following fillings, stainless steel crown placement and extractions (Berry et al 1973) and restorative treatment under general anaesthesia (Lockhart et al 2004). Although these are, in general, supportive of the present findings, interpretation is difficult because the samples taken were following a series of procedures which included anaesthetic induction, assumed placement of rubber dam although this was not stated, conservative dental procedures and occasional extractions. This makes causation of the post-procedure bacteraemia difficult because it could have been any one of the procedures, or a combination of them all, which caused the bacteraemia.

The approach in this study, which was to take a blood sample 30 seconds following the dento-gingival manipulative procedure, carried out as an isolated procedure, and compared with a baseline blood sample, provides robust results for interpretation. Bacteraemia detected in this way can be attributed to the dental procedure investigated. The placement of a rubber dam, matrix band and wedge and gingival retraction cord resulted in a significantly greater prevalence and intensity of bacteraemia compared to baseline in this study. It is important to add these procedures to those recommended for antibiotic prophylaxis in patients at risk of developing IE.

It is also important to note that odontogenic bacteraemia may be a risk for immunocompromised individuals. These include patients with haematologic and non-haematologic malignancies, immune deficiency syndromes, renal failure requiring dialysis, hepatic cirrhosis and patients taking cytotoxic or immunosuppressive medication (Reimer et al 1997). Recent reports have shown that the oral cavity was the source of streptococcal bacteraemia in immunocompromised patients (Richard et al 1995; Kennedy et al 2000; Kennedy et al 2003). The complications of streptococcal bacteraemia in immunocompromised patients are serious. They include acute respiratory distress, pneumonia and septic shock, resulting in significant morbidity and mortality (Richard et al 1995; Marron et al 2000).

Identification of isolates from bacteraemia is important to understand the pathophysiological processes involved and the accuracy of diagnosis. There are a wide range of microorganisms that may be detected from odontogenic bacteraemia. Using recent molecular techniques, 16S rRNA and *sodA* gene sequencing, the most frequently isolated bacteria following dento-gingival manipulation in this work were *Streptococcus* and *Staphylococcus* spp. These bacteria account for the majority of cases of IE (Bayliss et al 1983b; Hollanders et al 1988; Drangsholt 1998; Karchmer 1999; Hoen et al 2002). Other bacteria implicated in IE have also been isolated. These include *Rothia* spp., *Actinomyces* spp., *Propionibacterium* spp., *Neisseria* spp., *Haemophilus* spp. and *Micrococcus* spp.

It is hoped that the techniques used in this study will be further developed to speed and improve diagnosis. For example, although *sodA* gene sequencing is a useful and effective method for speciation of streptococci and CNS, there are several drawbacks that limit its routine use in clinical laboratories. *SodA* sequences are not available for all bacteria. As a result they cannot be applied to specific groups of bacteria unless corresponding sequence databases are enlarged. In addition, access to expensive and complex sequencing facilities is restricted to advanced molecular laboratories. These facilities are not generally available in hospitals and laboratories, especially in less developed countries.

It is also technically difficult to PCR and sequence bacterial DNA or RNA directly from blood (Ley et al 1998; Rothman et al 2002). It is therefore necessary to culture the organisms using conventional techniques. This translates as extended time required for bacterial identification. The ideal would be to develop molecular techniques so that different bacteria could be identified immediately from blood. This would also enable the identification of 'uncultivable' bacteria.

Further work:

1. Development of molecular techniques for identifying bacteria directly from blood.
2. Development and assessment of whole genome AFLP analysis for identification of streptococci.
3. The effect of a chlorhexidine mouthwash in reducing the bacterial load and the magnitude of bacteraemia.
4. The prevalence, intensity and identity of bacteraemia following other procedures and homecare such as toothbrushing and flossing.
5. Further work on the antibiotic sensitivity of oral bacteria and the transfer of resistance between species in the oral cavity. This is particularly relevant because of increasing antibiotic resistance.

REFERENCES

Abercrombie GF & Scott WM (1928) A case of infective endocarditis due to *Streptococcus mutans*. Lancet, ii: 697-699.

Adnan S, Li N, Miura H, Hashimoto Y, Yamamoto H & Ezaki T (1993) Covalently immobilized DNA plate for luminometric DNA-DNA hybridization to identify viridans streptococci in under 2 hours. FEMS Microbiol Lett, 106: 139-142.

Agvald-Öhman C, Lund B & Edlund C (2004) Multi-resistant coagulase-negative staphylococci disseminate frequently between intubated patients in a multidisciplinary intensive care unit. Critical Care, 8: 42-47.

Ahmet Z, Warren M & Houang ET (1995) Species identification of members of the *Streptococcus milleri* group isolated from the vagina by ID 32 Strep system and differential phenotypic characteristics. J Clin Microbiol, 33: 1592-1595.

Al Karaawi ZM, Lucas VS, Gelbier M & Roberts GJ (2001) Dental procedures in children with severe congenital heart disease: a theoretical analysis of prophylaxis and non-prophylaxis procedures. Heart, 85: 66-68.

Altman DG (1991) Practical statistics for medical research. Chapman & Hall, London.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. J Mol Biol, 215: 403-410.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res, 25: 3389-3402.

Andrewes RW & Horder TJ (1906) A study of the streptococci pathogenic for man. Lancet, ii: 708-713.

Aracil B, Miñambras M, Otes J, Torres C, Gomez-Garces JL & Alos J (2001) High prevalence of erythromycin-resistant and clindamycin-susceptible (M-phenotype) viridans group streptococci from pharyngeal samples: a reservoir of *mef* genes in commensal bacteria. J Antimicrob Chemother, 48: 592-594.

Archer GL & Scott J (1990) Conjugative transfer genes in staphylococcal isolates from the United States. Antimicrob Agent Chemother, 35: 2500-2504.

Archer GL & Climo MW (1994) Antimicrobial susceptibility of coagulase-negative staphylococci. Antimicrob Agent Chemother, 38: 2231-2237.

Ascher DP, Zbick C & White C (1991) Infections due to *Stomatococcus mucilaginosus*: 10 cases and review. Rev Infect Dis, 13: 1048-1052.

Auckenthaler R, Ilstrup DM & Washington JA (1982) Comparison of recovery of organisms from blood cultures diluted 10% (volume/volume) and 20% (volume/volume). J Clin Microbiol, 15: 860-864.

Augustine T, Thirunavukkarasu D, Bhat BV & Bhatia BD (1994) *Aerococcus viridans* endocarditis. Case report. Indian Pediatr, 31: 599-601.

Babaahmady KG, Marsh PD, Challacombe SJ & Newman HN (1997) Variations in the predominant cultivable microflora of dental plaque at defined subsites on approximal toothsurfaces in children. Arch Oral Biol, 42: 101-111.

Baddour LM, Christensen GD & Hester MG (1984) Production of experimental endocarditis by coagulase-negative staphylococci: variability in species virulence. J Infect Dis, 1: 721-727.

Baddour LM (1994) Virulence factors among Gram-positive bacteria in experimental endocarditis. Infect Immun, 62: 2143-2148.

Bahn SL, Goveia G, Bitterman P & Bahn AN (1978) Experimental endocarditis induced by dental manipulation and oral streptococci. Oral Surg Oral Med Oral Pathol, 45: 549-559.

Baker CN, Stocker SA, Culver DH & Thornsberry C (1991) Comparison of the E test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. J Clin Microbiol, 29: 533-538.

Baldo S (2003) The validity of a new index of plaque and gingivitis assessment and its use in children. MClinDent Thesis, University of London.

Banders JC & Dariouiche RO (1992) *Staphylococcus capitis* endocarditis: a new cause of an old disease. Clin Infect Dis, 14: 366-367.

Bannerman TL, Wadiak DL & Kloos WE (1991) Susceptibility of *Staphylococcus* species and subspecies to teicoplanin. Antimicrob Agent Chemother, 35: 1919-1922.

Bannerman TL, Kleeman KT & Kloos WE (1993) Evaluation of the Vitek systems Gram-Positive Identification Card for species identification of coagulase-negative staphylococci. J Clin Microbiol, 31: 1322-1325.

Bannister JV, Bannister WH & Rotilio G (1987) Aspects of the structure, function and applications of superoxide dismutase. CRC Rev Biochem, 22: 111-180.

Barco CT (1991) Prevention of infective endocarditis: a review of the medical and dental literature. J Periodontol, 62: 510-523.

Barker KF (1999) Antibiotic resistance: a current prespective. Br J Clin Pharmacol, 48: 109-124.

Bascomb S & Manafi M (1998) Use of enzyme tests in characterization and identification of aerobic and facultatively anaerobic Gram-positive cocci. Clin Microbiol Rev, 11: 318-340.

Baumgartner JC, Heggers JP & Harrison JW (1976) The incidence of bacteremias related to endodontic procedures. I. Nonsurgical endodontics. J Endod, 2: 135-140.

Bayer AS, Bolger AF, Taubert KA, Wilson W, Steckelberg J, Karchmer AW, Levison M, Chambers HF, Dajani AS, Gewitz MH, Newburger JW, Gerber MA, Shulman ST, Pallasch TJ, Gage TW & Ferrieri PL (1998) Diagnosis and management of infective endocarditis and its complications. Circulation, 98: 2936-2948.

Bayliss R, Clarke C, Oakley CM, Somerville W & Whitfield AG (1983a) The teeth and infective endocarditis. Br Heart J, 50: 506-512.

Bayliss R, Clarke C, Oakley CM, Somerville W, Whitfield AG & Young SE (1983b) The microbiology and pathogenesis of infective endocarditis. Br Heart J, 50: 513-519.

Bayliss R, Clarke C, Oakley CM, Somerville W, Whitfield AG & Young SE (1986) Incidence, mortality and prevention of infective endocarditis. J R Coll Physicians Lond, 20: 15-20.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE & Griffen AL (2002) Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol, 40: 1001-1009.

Behrman RE & Vaughan VC (1987) Cardiovascular disease In: Nelson's Textbook of Pediatrics. 13th ed, R. E. Behrman & V. C. Vaughan, Eds., W.B. Saunders Company, Philadelphia, PA, pp. 1010-1032.

Beighton D, Russell RR & Hayday H (1981) The isolation of characterization of *Streptococcus mutans* serotype h from dental plaque of monkeys (*Macaca fascicularis*). J Gen Microbiol, 124: 271-279.

Beighton D, Hardie JM & Whiley RA (1991a) A scheme for the identification of viridans streptococci. J Med Microbiol, 35: 367-372.

Beighton D, Russell RRB & Whiley RA (1991b) A simple biochemical scheme for the differentiation of *Streptococcus mutans* and *Streptococcus sobrinus*. Caries Res, 25: 174-178.

Beighton D, Carr AD & Oppenheim BA (1994) Identification of viridans streptococci associated with bacteraemia in neutropenic cancer patients. J Med Microbiol, 40: 202-204.

Bender IB & Pressman AB (1945) Factors in dental bacteraemia. J Am Dent Assoc, 32: 836-853.

Bender IB, Seltzer S & Yermish MA (1960) The incidence of bacteraemia in endodontic manipulation: preliminary report. Oral Surg Oral Med Oral Pathol, 13: 353-360.

Bender IB, Seltzer S, Meloff G & Pressman RS (1961) Conditions affecting sensitivity of techniques for detection of bacteremia. J Dent Res, 40: 951-959.

Bender IB, Seltzer S, Tashman S & Meloff G (1963) Dental procedures in patients with rheumatic heart disease. Oral Surg Oral Med Oral Pathol, 16: 466-473.

Bender IB, Naidorf IJ & Garvey GJ (1984) Bacterial endocarditis: a consideration for physician and dentist. J Am Dent Assoc, 109: 415-420.

Bender IB & Montgomery S (1986) Nonsurgical endodontic procedures for the patient at risk for infective endocarditis and other systemic disorders. J Endod, 12: 400-407.

Bensing BA, Rubens CE & Sullam PM (2001) Genetic loci of *Streptococcus mitis* that mediate binding to human platelets. Infect Immun, 69: 1373-1380.

Berry FA, Yarbrough S, Yarbrough N, Russell CM, Carpenter MA & Hendley JO (1973) Transient bacteremia during dental manipulation in children. Pediatrics, 51: 476-479.

Bhanji S, Williams B, Sheller B, Elwood T & Mancl L (2002) Transient bacteremia induced by toothbrushing a comparison of the Sonicare toothbrush with a conventional toothbrush. Pediatr Dent, 24: 295-299.

Biavasco F, Vignaralo C & Varaldo PE (2000) Glycopeptide resistance in coagulase-negative staphylococci. Eur J Clin Microbiol Infect Dis, 19: 403-417.

Binder D, Zbinden R, Widmer U, Opravil M & Krause M (1997) Native and prosthetic valve endocarditis caused by *Rothia dentocariosa*: diagnostic and therapeutic considerations. Infection, 25: 22-26.

Bitar FF, Jawdi RA, Dbaibo GS, Yunis KA, Gharzeddine W & Obeid M (2000) Paediatric infective endocarditis: 19-year experience at a tertiary care hospital in a developing country. Acta Paediatr, 89: 427-430.

Bochud P-Y, Eggiman Ph, Calandra Th, Van Melle G, Saghafi L & Francioli P (1994) Bacteremia due to viridans streptococcus in neutropenic patients with cancer: clinical spectrum and risk factors. Clin Infect Dis, 18: 25-31.

Boddinghaus B, Wolters J, Heikens W & Bottger EC (1990) Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol Lett, 70: 197-204.

Bor DH, Rose RM, Modlin JF, Weintraub R & Friedland GH (1983) Mediastinitis after cardiovascular surgery. Rev Infect Dis, 5: 885-897.

Bosshard PP, Abel S, Altwegg M, Böttger EC & Zbinden R (2004) Comparison of conventional and molecular methods for identification of aerobic catalase-negative Gram-positive cocci in the clinical laboratory. J Clin Microbiol, 42: 2065-2073.

Boudewijns M, Magerman K, Verhaegen J, Debrock G, Peetermans WE, Donkersloot P, Mewis A, Peeters V, Rummens JL & Cartuyvels R (2003) *Rothia dentocariosa*, endocarditis and mycotic aneurysms: case report and review of the literature. Clin Microbiol Infect, 9: 222-229.

Bourgault A & Gauvreau L (1983) Antimicrobial susceptibilities of coagulase-negative staphylococci isolated from urinary infections. Antimicrob Agent Chemother, 23: 793-795.

Bowden GH, Hardie J & Slack G (1975) Microbial variations in approximal dental plaque. Caries Res, 9: 253-277.

Bowden GH, Ekstrand J, McNaughton B & Challacombe SJ (1990) The association of selected bacteria with the lesions of root caries. Oral Microbiol Immunol, 5: 346-351.

Bridge PD & Sneath PHA (1982) *Streptococcus gallinarum* sp. nov. and *Streptococcus oralis* sp. nov. Int J Syst Evol Microbiol, 32: 410-415.

Broeren SA & Peel MM (1984) Endocarditis caused by *Rothia dentocariosa*. J Clin Pathol, 37: 1298-1300.

Brook I & Frazier EH (1993) Anaerobic osteomyelitis and arthritis in a military hospital – a 10 year experience. Am J Med, 94: 21-28.

Burket LW & Burn CG (1937) Bacteraemias following dental extraction. Demonstration of source of bacteria by means of a non pathogen (*Serratia marcesens*). J Dent Res, 16: 521-530.

Cabell CH, Jollis JG, Peterson GE, Corey GR, Anderson DJ, Sexton DJ, Woods CW, Reller LB, Ryan T & Fowler VG, Jr. (2002) Changing patient characteristics and the effect on mortality in endocarditis. Arch Intern Med, 162: 90-94.

Campoli-Richards DM, Brogden RN & Faulds D (1990) Teicoplanin: a review of its antibacterial activity, pharmacokinetic properites and therapeutic potential. Drugs, 40: 449-486.

Caputo GM, Archer GL, Calderwood SB, Dinubile MJ & Karchmer AW (1987) Native valve endocarditis due to coagulase-negative staphylococci: clinical and microbiologic features. Am.J.Med., 83: 619-625.

Carey RB (1984) Clinical comparison of the Isolator 1.5 microbial tube and the BACTEC radiometric system for detection of bacteremia in children. J Clin Microbiol, 19: 634-638.

Carmona IT, Diz DP & Scully C (2002) An update on the controversies in bacterial endocarditis of oral origin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 93: 660-670.

Carratalá J, Alcaide F, Fernandez-Sevilla A, Corbella X, Linares J & Gudiol F (1995) Bacteraemia due to viridans streptococci that are highly resistant to penicillin: increase among neutropenic patients with cancer. Clin Infect Dis, 20: 1169-1173.

Cates JE & Christie RV (1951) Subacute bacterial endocarditis. Quart J Med, 78: 93-130.

Cato E, Moore W, Nygaard G & Holdemann L (1984) *Actinomyces meyeri* sp. nov., specific epithet rev. Int J Syst Bacteriol, 34: 487-489.

Cercenado E, García-Leoni ME, Díaz MD, Sánchez-Carrillo C, Catalán P, Bernaldo de Quirós JCL & Bouza E (1996) Emergence of teicoplanin-resistant coagulase-negative staphylococci. J Clin Microbiol, 34: 1765-1768.

Chang HH, Lu C, Hsueh p, Wu M, Wang J & Huang L (2002) Endocarditis caused by *Abiotrophia defectiva* in children. Pediatr Infect Dis J, 21: 697-700.

Chang S, Hsieh W & Liu C (2000) High prevalence of antibiotic resistance of common pathogenic bacteria in Taiwan. Diagn Microbiol Infect Dis, 36: 107-112.

Charfreitag O, Collins MD & Stackerbrandt E (1988) Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int J Syst Bacteriol, 38: 354-357.

Chemlal K, Huys G, Fonteyne PA, Vincent V, Lopez AG, Rigouts L, Swings J, Meyers WM & Portaels F (2001) Evaluation of PCR-restriction profile analysis and IS*2404* restriction fragment length polymorphism and amplified fingerprinting for identification and typing of *Mycobacterium ulcerans* and *M. marinum*. J Clin Microbiol, 39: 3272-3278.

Cherubin CE & Neu HC (1971) Infective endocarditis at the Presbyterian Hospital in New York City from 1938-1967. Am J Med, 51: 83-96.

Chesneau O, Aubert S, Morvan A, Guesdon JL & El Solh N (1992) Usefulness of the ID32 Staph system and a method based on rRNA gene restriction site polymorphism analysis for species and subspecies identification of staphylococcal clinical isolates. J Clin Microbiol, 30: 2346-2352.

Chokephaibulkit K, Sitthitrai P, Wanprapa N, Chearskul S, Srifuengfung S, Pingwang B & Dhiraputra C (1999) Comparison of BACTEC automated blood culture system and conventional system in hospitalized pediatric patients. J Med Assoc Thai, 82: 1011-1016.

Choo MH, Holmes DR, Gersch BJ, Maloney JD, Meredith J, Pluth JR & Trusty J (1981) Permanent pacemaker infections: characterization and management. Am J Cardiol, 48: 559-564.

Ciantar M, Spratt DA, Newman HN & Wilson M (2001) *Capnocytophaga granulosa* and *Capnocytophaga haemolytica*: novel species in subgingival plaque. J Clin Periodontol, 28: 701-705.

Clarke JK (1924) On the bacterial factor in the aetiology of dental caries. Br J Exp Pathol, 5: 141-147.

Clarridge JE & Zhang Q (2002) Genotypic diversity of clinical *Actinomyces* species: phenotype, source, and disease correlation among genospecies. J Clin Microbiol, 40: 3442-3448.

Cobe M (1954) Transitory bacteraemia. Oral Surg Oral Med Oral Pathol, 7: 609-615.

Collins MD, Ash C, Farrow JAE, Wallblanks S & Williams AM (1989) 16S rRNA sequence analysis of lactococci and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. nov. J Appl Bacteriol, 67: 453-460.

Collins MD, Aguirre M, Facklam RR, Shallcross J & Williams AM (1992) *Globicatella sanguis* gen. nov., a new Gram-positive catalase-negative bacterium from human sources. J Appl Bacteriol, 73: 433-437.

Collins MD, Falsen E, Lemozy E, Akervall E, Sjoden B & Lawson PA (1997) Phenotypic and phylogenetic characterization of some *Globicatella*-like organisms from human sources: description of *Facklamia hominis* gen. nov., sp. nov. Int J Syst Bacteriol, 47: 880-882.

Collins MD, Jovita MA, Hutson RA, Falsen E, Nikolaitchouk N & Facklam RR (1999a) *Dolosicoccus paucivorans* gen. nov. sp., isolated from human blood. Int J Syst Bacteriol, 49: 1439-1442.

Collins MD, Lawson PA, Monasterio R, Falsen E, Sjoden B & Facklam RR (1999b) *Ignavigranum ruoffiae* sp. nov. isolated from human clinical specimens. Int J Syst Bacteriol, 49: 97-101.

Collins MD, Hoyles L, Kalfas S, Sundquist G, Monsen T, Nikolaitchouk N & Falsen E (2000) Characterization of *Actinomyces* isolates from infected root canals of teeth: description of *Actinomyces radicidentis* sp. nov. J Clin Microbiol, 38: 3399-3403.

Collins MD & Lawson PA (2000) The genus *Abiotrophia* (Kamaura et al.) is not monophyletic: proposal of *Granulicatella* gen. nov., *Granulicatella adjacens* comb. nov., *Granulicatella elegans* comb. nov. and *Granulicatella balaenopterae* comb. nov. Int J Syst Bacteriol, 50: 365-369.

Colman G & Williams REO (1972) Taxonomy of some human viridans streptococci In: Streptococci and streptococcal diseases L. W. Wannamaker & J. M. Matsen, Eds., Academic Press, Inc., New York, pp. 282-299.

Conville PS, Fischer SH, Cartwright CP & Witebsky FG (2000) Identification of *Nocardia* species by restriction endonuclease analysis of an amplified portion of the 16S rRNA gene. J Clin Microbiol, 38: 158-164.

Corson MA, Postlethwaite KP & Seymour RA (2001) Are dental infections a cause of brain abscess? Case report and review of the literature. Oral Dis, 7: 61-65.

Coudron PE, Markowitz SM, Mohanty LB, Schatzki PF & Payne JM (1987) Isolation of *Stomatococcus mucilaginosus* from drug user with endocarditis. J Clin Microbiol, 25: 1359-1363.

Coulter WA, Coffey A, Saunders IDF & Emmerson AM (1990) Bacteremia in children following dental extraction. J Dent Res, 69: 1691-1695.

Cowan ST & Steel KJ (1993) Manual for the identification of medical bacteria. 3rd ed, Cambridge University Press, Cambridge.

Coward K, Tucker N & Darville T (2003) Infective endocarditis in Arkansan children from 1990 through 2002. Pediatr Infect Dis J, 22: 1048-1052.

Coykendall AL (1971) Genetic heterogeneity in *Streptococcus mutans*. J Bacteriol, 106: 192-196.

Coykendall AL (1974) Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. J Gen Microbiol, 83: 327-338.

Coykendall AL, Specht PA & Samol HH (1974) *Streptococcus mutans* in a wild, sucrose-eating rat population. Infect Immun, 10: 216-219.

Coykendall AL & Specht PA (1975) DNA base sequence homologies among strains of *Streptococcus sanguis*. J Gen Microbiol, 91: 92-98.

Cremieux A-C, Saleh-Mghir A, Vallois J-M, Muffat-Joly M, Devine C, Pocidalo J-J & Carbon C (1993) Influence of the pre-treatment duration of infection on the efficacies of various antibiotic regimens in experimental streptococcal endocarditis. J Antimicrob Chemother, 32: 843-852.

Dahlen G & Wikstrom M (1995) Occurrence of enteric rods, staphylococci and Candida in subgingival samples. Oral Microbiol Immunol, 10: 42-46.

Dajani AS, Taubert KA, Wilson W, Bolger AF, Bayer A, Ferrieri PL, Gewitz MH, Shulman ST, Nouri S, Newburger JW, Hutto C, Pallasch TJ, Gage TW, Levison ME, Peter G & Zuccaro G (1997) Prevention of bacterial endocarditis. Recommendations by the American Heart Association. JAMA, 277: 1794-1801.

Daly CG, Mitchell DH, Highfield JE, Grossberg DE & Stewart D (2001) Bacteremia due to periodontal probing: a clinical and microbiological investigation. J Periodontol, 72: 210-214.

Dan M, Marien GJR & Goldsand G (1984) Endocarditis caused by *Staphylococcus warneri* on a normal aortic valve following vasectomy. Can Med Assoc J, 131: 211-213.

Daniels C, Schoors D & Van Cramp G (2003) Native valve endocarditis with aorta-to-left atrial fistula due to *Corynebacterium amycolatum*. Eur J Echocardiogr, 4: 68-70.

Dankert JD, van Der Werff J, Zaat SAJ, Joldersma W, Klein D & Hess J (1995) Involvement of bactericidal factors from thrombin stimulated platelets in clearance of adherent viridans streptococci in experimental endocarditis. Infect Immun, 63: 663-671.

Das M, Badley AD, Cockerill FR, Steckelberg JM & Wilson WR (1997) Infective endocarditis caused by HACEK microorganisms. Annu Rev Microbiol, 48: 25-33.

De Leo AA, Schoenknecht FD, Anderson MW & Peterson JC (1974) The incidence of bacteremia following oral prophylaxis on pediatric patients. Oral Surg Oral Med Oral Pathol, 37: 36-45.

Debelian GJ, Olsen I & Tronstad L (1992) Profiling of *Propionibacterium acnes* recovered from root canal and blood during and after endodontic treatment. Endod Dent Traumatol, 8: 248-254.

Debelian GJ, Olsen I & Tronstad L (1995) Bacteremia in conjunction with endodontic therapy. Endod Dent Traumatol, 11: 142-149.

Debelian GJ, Eribe ER, Olsen I & Tronstad L (1997) Ribotyping of bacteria from root canal and blood of patients receiving endodontic therapy. Anaerobe, 3: 237-243.

Decousser JW, Pina P, Picot F, Delalande C, Pangon B, Courvalin P & Allouch P (2003) Frequency of isolation and antimicrobial susceptibility of bacterial pathogens from patients with bloodstream infections: a French prospective national survey. J Antimicrob Chemother, 51: 1213-1222.

Del' Alamo L, Cereda RF, Tosin I, Miranda A & Sader HS (1999) Antimicrobial susceptibility of coagulase-negative staphylococci and characterization of isolates with reduced susceptibility to glycopeptides. Antimicrob Agent Chemother, 34: 185-191.

Delahaye F & Gevigney G (2001) Should we give antibiotic prophylaxis against infective endocarditis in all cardiac patients, whatever the type of dental treatment? Heart, 85: 9-10.

Delcenseri V, Bechoux N, Leonard T & China B (2004) Discrimination between *Bifidobacterium* species of human and animal origin by PCR-restriction fragment length polymorphism. J Food Prot, 67: 1284-1288.

Devlin RK, Andrews MM & von Reyn CF (2004) Recent trends in infective endocarditis: influence of case definitions. Curr Opin Cardiol, 19: 134-139.

Devriese LA, Pot B, Vandamme P, Kersters K, Collins MD, Alvarez N, Haesebrouck F & Hommez J (1997) *Streptococcus hyovaginalis* sp. nov. and *Streptococcus thoraltensis* sp. nov., from the genital tract of sows. Int J Syst Evol Microbiol, 47: 1073-1077.

Dhawan A, Grover A, Marwaha RK, Khattri HN, Anand IS, Kumar L, Walia BN & Bidwai PS (1993) Infective endocarditis in children: profile in a developing country. Ann Trop Paediatr, 13: 189-194.

Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones N & Beach M (2001) Survey of infections due to *Staphylococcus* species: frequency of occurence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe and the Western Pacific region for the SENTRY antimicrobial surveillance program, 1997-1999. Clin Infect Dis, 32 (Suppl 2): S114-S132.

Doern GV, Ferraro MJ, Brueggemann AB & Ruoff KL (1996) Emergence of high rates of antimicrobial resistance amongs viridans group streptococci in the United States. Antimicrob Agent Chemother, 40: 891-894.

Dorn GL, Land GA & Wilson GE (1979) Improved blood culture technique based on centrifugation: clinical evaluation. J Clin Microbiol, 9: 391-396.

Douglas CP, Siarakas S & Gottlieb T (1994) Evaluation of E test as a rapid method for determining MIC's for nutritionally variant streptococci. J Clin Microbiol, 32: 2318-2320.

Douglas CW, Heath J, Hampton KK & Preston FE (1993) Identity of viridans streptococci isolated from cases of infective endocarditis. J Med Microbiol, 39: 179-182.

Douglas CW (1994) Possible pathogenic mechanisms of oral streptococci. Microb Ecol Health Dis, 7: 175-177.

Doyuk E, Ormerod OJ & Bowler CJW (2002) Native valve endocarditis due to *Streptococcus vestibularis* and *Streptococcus oralis*. J Infect, 45: 39-41.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP & Raoult D (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol, 38: 3623-3630.

Drangsholt MT (1998) A new causal model of dental diseases associated with endocarditis. Ann Periodontol, 3: 184-196.

Droz D, Koch L, Lenain A & Michalski H (1997) Bacterial endocarditis: results of a survey in a children's hospital in France. Br Dent J, 183: 101-105.

Drucker DB & Jolly M (1971) Sensitivity of oral micro-organisms to antibiotics. Br Dent J, 131: 442-444.

Durack DT, Beeson PB & Petersdorf RG (1973) Experimental bacterial endocarditis. 3. Production and progress of the disease in rabbits. Br J Exp Pathol, 54: 142-151.

Durack DT & Towns M (1998) Diagnosis and managment of infective endocarditis In: Evidence based cardiology. S. Yusuf et al., Eds., BMJ Publishing Grp, London, pp. 884-904.

Dyson C, Barnes RA & Harrison GA (1999) Infective endocarditis: an epidemiological review of 128 episodes. J Infect, 38: 87-93.

Ellen RP (1976) Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in the human oral cavity. Infect Immun, 14: 1119-1124.

Elliott RH & Dunbar JM (1968) Streptococcal bacteraemia in children following dental extractions. Arch Dis Child, 43: 451-453.

Erverdi N, Kadir T, Ozkan H & Acar A (1999) Investigation of bacteremia after orthodontic banding. Am J Orthod Dentofacial Orthop, 116: 687-690.

Etienne J, Fleurette J, Ninet JF, Favet P & Gruer LD (1986) Staphylococcal endocarditis after dental extraction. Lancet, 30: 511-512.

Etienne J, Brun Y, El Solh N, Delorme V, Mouren C, Bes M & Fleurette J (1988) Characterization of clinically significant isolates of *Staphylococcus epidermidis* from patients with endocarditis. J Clin Microbiol, 26: 613-617.

Etienne J & Eykyn SJ (1990) Increase in native valve endocarditis caused by coagulase-negative staphylococci: an Anglo-French clinical and microbiological study. Br Heart J, 64: 381-384.

Everett ED & Hirschmann JV (1977) Transient bacteremia and endocarditis prophylaxis. A review. Medicine, 56: 61-77.

Ezaki T, Hashimoto Y, Takeuchi N, Yamamoto H, Liu S, Miura H, Matsui K & Yabuuchi E (1988) Simple genetic method to identify viridans group streptococci by colorimetric dot hybridization and fluorometric hybridization in microdilution wells. J Clin Microbiol, 26: 1708-1713.

Facklam RR (1977) Physiological differentiation of viridans streptococci. J Clin Microbiol, 5: 184-201.

Facklam RR (1984) The major differences between the American and British Streptococcus taxonomy schemes with special reference to *Streptococcus milleri*. Eur J Clin Microbiol, 3: 91-93.

Facklam RR (2002) What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev, 15: 613-630.

Farrington FH (1973) The incidence of transient bacteremia following pulpotomies on primary teeth. ASDC J Dent Child, 40: 175-184.

Feder HM Jr, Roberts JC, Salazar JC, Leopold HB & Toro-Salazar O (2003) HACEK endocarditis in infants and children: two cases and a literature review. Pediatr Infect Dis J, 22: 557-562.

Fefer P, Raveh D, Rudensky B, Schlesinger Y & Yinnon AM (2002) Changing epidemiology of infective endocarditis: a retrospective survey of 108 cases, 1990-1999. Eur J Clin Microbiol Infect Dis, 21: 432-437.

Felder RS, Nardone D & Palac R (1992) Prevalence of predisposing factors for endocarditis among an elderly institutionalized population. Oral Surg Oral Med Oral Pathol, 73: 30-34.

Felix JE, Rosen S & App GR (1971) Detection of bacteremia after the use of an oral irrigation device in subjects with periodontitis. J Periodontol, 42: 785-789.

Fernicola DJ & Roberts WC (1993) Clinicopathologic features of active infective endocarditis isolated to the native mitral valve. Am J Cardiol, 71: 1186-1197.

Ferrieri PL, Gewitz MH, Gerber MA, Newburger JW, Dajani AS, Shulman ST, Wilson W, Bolger AF, Bayer A, Levison ME, Pallasch TJ, Gage TW & Taubert KA (2002) Unique features of infective endocarditis in childhood. Circulation, 105: 2115-2126.

Fiehn NE, Gutschik E, Larsen T & Bangsborg JM (1995) Identity of streptococcal blood isolates from two patients with infective endocarditis. J Clin Microbiol, 33: 1399-1401.

Fleming P, Feigal RJ, Kaplan EL, Liljemark WF & Little JW (1990) The development of penicillin-resistant oral streptococci after repeated penicillin prophylaxis. Oral Surg Oral Med Oral Pathol, 70: 440-444.

Fluckiger U, Moreillon P, Blaser J, Bickle M, Glauser MP & Francioli P (1994) Simulation of amoxicillin pharmacokinetics in humans for the prevention of streptococcal endocarditis in rats. Antimicrob Agent Chemother, 38: 2846-2849.

Fluit AC, Verhoef J & Schmitz FJ (2001) Frequency of isolation and antimicrobial resistance of Gram-negative and Gram-positive bacteria from patients in intensive care units of 25 European university hospitals participating in the European arm of the SENTRY Antimicrobial Surveillance Program 1997-1998. Eur J Clin Microbiol Infect Dis, 20: 617-625.

Fox GE & Woese CR (1975) The architecture of 5S rRNA and its relation to function. J Mol Evol, 6: 61-76.

Fox GE, Wisotzkey JD & Jurtshuk P, Jr. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Evol Microbiol, 42: 166-170.

Franco E, Saunders CP, Roberts GJ & Suwanprasit A (1996) Dental disease, caries related microflora and salivary IgA of children with severe congenital cardiac disease: an epidemiological and oral microbial survey. Pediatr Dent, 18: 228-235.

Freedman LR (1987) The pathogenesis of infective endocarditis. J Antimicrob Chemother, 20: 1-6.

Fritsche TR, Sader HS & Jones RN (2003) Comparative activity and spectrum of broad-spectrum ß-lactams (cefepime, ceftazidime, ceftriaxone, piperacillin/tazobactam) tested against 12,295 staphylococci and streptococci: report from the SENTRY antimicrobial surveillance program (North America: 2001-2002). Diagn Microbiol Infect Dis, 47: 435-440.

Garnier F, Gerbaud G, Courvalin P & Galimand M (1997) Identification of clinically relevant viridans group streptococci to the species level by PCR. J Clin Microbiol, 35: 2337-2341.

Gelbier M, Lucas VS, Zervou NE, Roberts GJ & Novelli V (2000) A preliminary investigation of dental disease in children with HIV infection. Int J Paediatr Dent, 10: 13-18.

Geraci JE & Wilson WR (1982) Symposium on infective endocarditis. III. Endocarditis due to Gram-negative bacteria. Report of 56 cases. Mayo Clin Proc, 57: 145-148.

Gersony WM & Hayes CJ (1977) Bacterial endocarditis in patients with pulmonary stenosis, aortic stenosis, or ventricular septal defect. Circulation, 56 (Suppl 1): I84-I87.

Gersony WM & Hordof AJ (1978) Infective endocarditis and diseases of the pericardium. Pediatr Clin North Am, 25: 831-846.

Geva T & Frand M (1988) Infective endocarditis in children with congenital heart disease: the changing spectrum, 1965-85. Eur Heart J, 9: 1244-1249.

Gill VJ, Selepak ST & Williams EC (1983) Species identification and antibiotic susceptibilities of coagulase-negative staphylococci isolated from clinical specimens. J Clin Microbiol, 18: 1314-1319.

Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP & Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J Clin Microbiol, 34: 818-823.

Goldstein EJC, Citron DM, Merriam CV, Warren YA, Tyrrell KL & Fernandez HT (2003) *In vitro* activities of daptomycin, vancomycin, quinupristin-dalfopristin, linezolid and five other antimicrobials against 307 Gram-positive anaerobic and 31 *Corynebacterium* clinical isolates. Antimicrob Agent Chemother, 47: 337-341.

Goldstein FW, Coutrot A, Seiffer A & Acar JF (1990) Percentages and distributions of teicoplanin- and vancomycin-resistant strains among coagulase-negative staphylococci. Antimicrob Agent Chemother, 34: 899-900.

Greenwood D & Slack RCB (2000) Antibiotic sensitivity testing and assay In: Antimicrobial chemotherapy. 4th ed, D. Greenwood, Ed., Oxford University Press, New York, pp. 101-117.

Gruer LD, Bartlett R & Ayliffe GAJ (1984) Species identification and antimicrobic sensitivity of coagulase-negative staphylococci from CAPD peritonitis. J Antimicrob Chemother, 13: 577-583.

Guiot HFL, Corel LJA & Vossen JMJ (1994) Prevalence of penicillin-resistant viridans streptococci in healthy children and in patients with malignant haematological disorders. Eur J Clin Microbiol, 13: 645-650.

Gunn B (1989) Comparative virulence of human isolates of coagulase-negative staphylococci tested in an infant mouse weight retardation model. J Clin Microbiol, 27: 507-511.

Günthard H, Hany A, Turina M & Wüst J (1994) *Propionibacterium acnes* as a cause of aggressive aortic valve endocarditis and importance of tissue grinding: case report and review. J Clin Microbiol, 32: 3043-3045.

Guthof O (1956) Uber pathogene, vergrunende 'Streptokokken'. Streptokokkenbefunde bei dentogenen Abszessen und Ibfiltraten im Beriech der Mundhohle. Zentralblatt fur Backteriologie, Parasitenkinde, Infektionskankheiten und Hygiene, 166: 553-564.

Gutschik E (1976) Endocarditis caused by *Actinomyces viscosus*. Scand J Infect Dis, 8: 271-274.

Gutschik E & Lippert S (1990) Dental procedures and endocarditis prophylaxis: experiences from 108 dental practices. Scand J Dent Res, 98: 144-148.

Hall G, Heimdahl A & Nord CE (1996a) Effects of prophylactic administration of cefaclor on transient bacteremia after dental extraction. Eur J Clin Microbiol Infect Dis, 15: 646-649.

Hall G, Nord CE & Heimdahl A (1996b) Elimination of bacteraemia after dental extraction: comparison of erythromycin and clindamycin for prophylaxis of infective endocarditis. J Antimicrob Chemother, 37: 783-795.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp, 41: 95-98.

Hamed KA (1998) Successful treatment of primary *Actinomyces viscosus* endocarditis with third-generation cephalosporins. Clin Infect Dis, 26: 211-212.

Handley PS, Carter PL, Wyatt JE & Hesketh LM (1985) Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. Infect Immun, 47: 217-227.

Hardie JM & Whiley RA (1994) Recent developments in streptococcal taxonomy : their relation to infections. Rev Med Microbiol, 5: 151-162.

Harris JC, Bryan RA, Lucas VS & Roberts GJ (2001) Dental disease and caries related microflora in children with dystrophic epidermolysis bullosa. Pediatr Dent, 23: 438-443.

Harvey WP & Capone MA (1961) Bacterial endocarditis related to cleaning and filling of teeth. Am J Cardiol, 7: 793-798.

Hashemi FB, Schutze GE & Mason EO, Jr (1996) Discrepencies between results by E test and standard microbroth dilution testing of *Streptococcus pneumoniae* for susceptibility to vancomycin. J Clin Microbiol, 34: 1546-1547.

Heimdahl A, Josefsson K, von Konow L & Nord CE (1985) Detection of anaerobic bacteria in blood cultures by lysis filtration. Eur J Clin Microbiol, 4: 404-407.

Heimdahl A, Hall G, Hedberg M, Sandberg H, Soder PO, Tuner K & Nord CE (1990) Detection and quantitation by lysis-filtration of bacteremia after different oral surgical procedures. J Clin Microbiol, 28: 2205-2209.

Henwood CJ, Livermore DM, Johnson AP, James D, Warner M & Gardiner A (2000) Susceptibility of Gram-positive cocci from 25 UK hospitals to antimicrobial agents including linezolid. J Antimicrob Chemother, 46: 931-940.

Hernandez JL, Calvo J, Sota R, Aguero J, Garcia-Palomo JD & Farinas MC (2001) Clinical and microbiological charactersitics of 28 patients with *Staphylococcus schleiferi* infection. Eur J Clin Microbiol Infect Dis, 20: 153-158.

Herzberg MC, Gong K, MacFarlane GD, Erickson PR, Soberay AH, Krebsbach PH, Manjula G, Schilling K & Bowen WH (1990) Phenotypic characterization of *Streptococcus sanguis* virulence factors associated with bacterial endocarditis. Infect Immun, 58: 515-522.

Herzberg MC, MacFarlane GD, Gong K, Armstrong NN, Witt AR & Erickson PR (1992) The platelet interactivity phenotype of *Streptococcus sanguis* influences the course of experimental endocarditis. Infect Immun, 60: 4809-4818.

Hess J, Dankert J & Durack D (1983a) Significance of penicillin tolerance *in vivo*: prevention of experimental *Streptococcus sanguis* endocarditis. J Antimicrob Chemother, 11: 555-564.

Hess J, Holloway Y & Dankert J (1983b) Incidence of postextraction bacteremia under penicillin cover in children with cardiac disease. Pediatrics, 71: 554-558.

Hillman JD, Andrews SW, Painter S & Stashenko P (1989) Adaptive changes in strains of *Streptococcus mutans* during colonization of the human oral cavity. Microb Ecol Health Dis, 2: 231-239.

Hockett RN, Loesche WJ & Sodeman TM (1977) Bacteraemia in asymptomatic human subjects. Arch Oral Biol, 22: 91-98.

Hoen B, Alla F, Selton-Suty C, Beguinot I, Bouvet A, Briancon S, Casalta JP, Danchin N, Delahaye F, Etienne J, Le Moing V, Leport C, Mainardi JL, Ruimy R & Vandenesch F (2002) Changing profile of infective endocarditis: results of a 1-year survey in France. JAMA, 288: 75-81.

Hoesley CJ & Cobbs CG (1999) Endocarditis at the millennium. J Infect Dis, 179 (Suppl 2): S360-S365.

Hohwy J & Kilian M (1995) Clonal diversity of the *Streptococcus mitis* biovar 1 population in the human oral cavity and pharynx. Oral Microbiol Immunol, 10: 19-25.

Hoke C & Vedros NA (1982) Taxonomy of the neisseriae: deoxyribonucleic acid base composition, interspecific transformation and dexoyribonucleic acid hybridization. Int J Syst Bacteriol, 32: 57-66.

Hollanders G, De Scheerder I, De Buyzere M, Ingels G, Bogaert S & Clement DL (1988) A six years review on 53 cases of infective endocarditis: clinical, microbiological and therapeutical features. Acta Cardiol, 43: 121-132.

Holmstrup P, Poulsen AH, Andersen L, Skuldbol T & Fiehn NE (2003) Oral infections and systemic diseases. Dent Clin North Am, 47: 575-598.

Hopkins RJ, Schwalbe RS & Donnenberge M (1992) Infections due to *Stomatococcus mucilaginosus*: report of two new cases and review. Clin Infect Dis, 14: 1264.

Horstkotte D, Follath F, Gutschik E, Lengyel M, Oto A, Pavie A, Soler-Soler J, Thiene G, von Graevenitz A, Priori SG, Garcia MA, Blanc JJ, Budaj A, Cowie M, Dean V, Deckers J, Fernandez BE, Lekakis J, Lindahl B, Mazzotta G, Morais J, Oto A, Smiseth OA, Lekakis J, Vahanian A, Delahaye F, Parkhomenko A, Filipatos G, Aldershvile J & Vardas P (2004) Guidelines on prevention, diagnosis and treatment of infective endocarditis executive summary; the task force on infective endocarditis of the European society of cardiology. Eur Heart J, 25: 267-276.

Houston S, Taylor D & Rennie R (1997) Prosthetic valve endocarditis due to *Veillonella dispar*: successful medical treatment following penicillin desensitization. Clin Infect Dis, 24: 1013-1014.

Huang MB, Baker CN, Banerjee S & Tenover FC (1992) Accuracy of the E test for determining antimicrobial susceptibilities of staphylococci, enterococci, *Campylobacter jejuni*, and Gram-negative bacteria resistant to antimicrobial agents. J Clin Microbiol, 30: 3243-3248.

Huebner J & Goldmann DA (1999) Coagulase-negative staphylococci: role as pathogens. Annu Rev Med, 50: 223-236.

Idigoras P, Valiente A, Iglesias L, Trieu-Cout P & Poyart C (2001) Meningitis due to *Streptococcus salivarius*. J Clin Microbiol, 39: 3017.

Ieven M, Verhoeven J, Pattyn SR & Goossens H (1995) Rapid and economical methods for species identification of clinically significant coagulase-negative staphylococci. J Clin Microbiol, 33: 1060-1063.

Ioannidou S, Tassios PT, Kotsovili-Tseleni A, Foustoukou M, Legakis NJ & Vatopoulos A (2001) Antibiotic resistance rates and macrolide resistance phenotypes of viridans group streptococci from the oropharynx of healthy Greek children. Int J Antimicrob Agents, 17: 195-201.

Isaacson JH & Grenko RT (1988) *Rothia dentocariosa* endocarditis complicated by brain abscess. Am J Med, 84: 352-354.

Ishikawa O (1980) Aerobic Gram-positive pleomorphic rods isolated from dental plaque and gingival crevice. Bull Tokyo Med Dent Univ, 27: 71-77.

Jackson MS, Bagg J, Gupta MN & Sturrock RD (1999) Oral carriage of staphylococci in patients with rheumatoid arthritis. Rheumatology, 38: 572-575.

Jaffray E (2003) *SodA* restriction digest and RFLP for streptococci (unpublished work).

Jarvis WR & Martone WJ (1992) Predominant pathogens in hospital infections. J Antimicrob Chemother, 29: 19-24.

Jenkinson HF & Lamont RJ (1997) Streptococcal adhesion and colonization. Crit Rev Oral Biol Med, 8: 175-200.

Johnson AP, Warner M, Broughton K, James D, Efsratiou A, George RC & Livermore DM (2001) Antibiotic susceptibility of streptococci and related genera causing endocarditis: analysis of UK reference laboratory referrals, January 1996 to March 2000. Br Med J, 322: 395-396.

Johnson AP, Henwood C, Mushtaq S, James D, Warner M & Livermore DM (2003) Susceptibility of Gram-positive bacteria from ICU patients in UK hospitals to antimicrobial agents. J Hosp Infect, 54: 179-187.

Johnson CM & Rhodes KH (1982) Pediatric endocarditis. Mayo Clin Proc, 57: 86-94.

Johnson DH, Rosenthal A & Nadas AS (1975) A forty-year review of bacterial endocarditis in infancy and childhood. Circulation, 51: 581-588.

Johnson JL, Moore LVH, Kaneko B & Moore WEC (1990) *Actinomyces georgiae* sp. nov., *Actinomyces gerencseriae* sp, nov. Designation of two genospecies of *Actinomyces naeslundii,* and inclusion of *A. naeslundii* serotypes II and III and *Actinomyces viscosus* serotype II in *A. naeslundii* genospecies 2. Int J Syst Bacteriol, 40: 273-286.

Jokinen MA (1970) Bacteraemia following dental extraction and its prophylaxis. Suom Hammaslaak Toim, 65: 69-100.

Kaditis AG, O'Marcaigh AS, Rhodes KH, Weaver AL & Henry NK (1996) Yield of positive blood cultures in pediatric oncology patients by a new method of blood culture collection. Pediatr Infect Dis J, 15: 615-620.

Kagan RL, Schuette WH, Zierdt CH & MacLowry JD (1977) Rapid automated diagnosis of bacteremia by impedance detection. J Clin Microbiol, 5: 51-57.

Kamma JJ, Diamanti-Kipioti A, Nakou M & Mitsis F (2000) Profile of subgingival microbiota in children with mixed dentition. Oral Microbiol Immunol, 15: 103-111.

Kanafani ZA, Mahfouz TH & Kanj SS (2002) Infective endocarditis at a tertiary care centre in Lebanon: predominance of streptococcal infection. J Infect, 45: 152-159.

Kanamoto T, Eifuku-Koreeda H & Inoue M (1996) Isolation and properties of bacteriolytic enzyme-producing cocci from the human mouth. FEMS Microbiol Lett, 144: 135-140.

Kaplan EL, Rich H, Gersony W & Manning J (1979) A collaborative study of infective endocarditis in the 1970s. Emphasis on infections in patients who have undergone cardiovascular surgery. Circulation, 59: 327-335.

Karchmer AW, Archer GL & Dismukes WE (1983) *Staphylococcus epidermidis* prosthetic valve endocarditis: microbiological and clinical observations as a guide to therapy. Ann Intern Med, 98: 447-455.

Karchmer AW (1999) Infective endocarditis In: Clinical infectious diseases. KR Root et al., Eds., Oxford University Press, Inc, New York, pp. 621-635.

Kawamura Y, Hou XG, Sultana F, Liu S, Yamamoto H & Ezaki T (1995a) Transfer of *Streptococcus adjacens* and *Streptococcus defectivus* to *Abiotrophia* gen. nov. as *Abiotrophia adiacens* comb. nov. and *Abiotrophia defectiva* comb. nov., respectively. Int J Syst Evol Microbiol, 45: 798-803.

Kawamura Y, Hou XG, Sultana F, Miura H & Ezaki T (1995b) Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Evol Microbiol, 45: 406-408.

Kawamura Y, Hou X, Sultana F, Hirose K, Miyake M, Shu S & Ezaki T (1998a) Distribution of *Staphylococcus* species among human clinical specimens and emended description of *Staphylococcus caprae*. J Clin Microbiol, 36: 2038-2042.

Kawamura Y, Hou XG, Todome Y, Sultana F, Hirose K, Shu SE, Ezaki T & Ohkuni H (1998b) *Streptococcus peroris* sp. nov. and *Streptococcus infantis* sp. nov., new members of the *Streptococcus mitis* group, isolated from human clinical specimens. Int J Syst Evol Microbiol, 48: 921-927.

Kawamura Y, Whiley RA, Shu SE, Ezaki T & Hardie JM (1999) Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. Microbiology, 145: 2605-2613.

Kawamura Y, Whiley RA, Zhao L, Ezaki T & Hardie JM (2000) Taxonomic study of "tufted mitior" strains of streptococci (*Streptococcus sanguinis* biotype 11); recognition of a new genospecies. Syst Appl Microbiol, 23: 245-250.

Kaye D (1994) Infective endocarditis In: Harrison's principles of internal medicine. 13th ed, Isselbacher et al., Eds., McGraw-Hill, New York, pp. 520-526.

Kellogg JA, Manzella JP & Bankert DA (2000) Frequency of low-level bacteremia in children from birth to fifteen years of age. J Clin Microbiol, 38: 2181-2185.

Kennedy HF, Morrison D, Kaufmann ME, Jackson MS, Bagg J, Gibson BE, Gemmell CG & Michie JR (2000) Origins of *Staphylococcus epidermidis* and *Streptococcus oralis* causing bacteraemia in a bone marrow transplant patient. J Med Microbiol, 49: 367-370.

Kennedy HF, Gemmel CG, Bagg J, Gibson BE & Michie JR (2001) Antimicrobial susceptibility of blood culture isolates of viridans streptococci: relationship to a change in empirical antibiotic therapy in febrile neutropenia. J Antimicrob Chemother, 47: 693-696.

Kennedy HF, Morrison D, Tomlinson D, Gibson BE, Bagg J & Gemmell CG (2003) Gingivitis and toothbrushes: potential roles in viridans streptococcal bacteraemia. J Infect, 46: 67-70.

Khairat O (1966) An effective antibiotic cover for the prevention of endocarditis following dental and other post-operative bacteraemias. J Clin Pathol, 19: 561-566.

Kidd SA, Rademeyer C, Roberts GJ, Lee PJ & Lucas VS (2002) Dental disease indices and caries-related microflora in children with glycogen storage disease. Int J Paediatr Dent, 12: 8-13.

Kikuchi K, Enari T, Minami S, Haruki Y, Shibata Y, Hasegawa H, Katahira J, Totsuka K & Shimuzu K (1994) Postantibiotic effects and postantibiotic sub-MIC effects of benzylpenicillin on viridans streptococci isolated from patients with infective endocarditis. J Antimicrob Chemother, 34: 687-696.

Kikuchi K, Enari T, Totsuka K & Shimizu K (1995) Comparison of phenotypic characteristics, DNA-DNA hybridization results, and results with a commercial rapid biochemical and enzymatic reaction system for identification of viridans group streptococci. J Clin Microbiol, 33: 1215-1222.

Kilian M, Mikkelsen M & Henrichsen J (1989) Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven, 1946), *Streptococcus oralis* (Bridge and Sneath 1982) and *Streptococcus mitis* (Andrews and Horder 1906). Int J Syst Evol Microbiol, 39: 471-484.

Kilpper-Balz R, Wenzig P & Schleifer KH (1985) Molecular relationships and classification of some viridans streptococci as *Streptococcus oralis* and emended description of *Streptococcus oralis* (Bridge and Sneath 1982). Int J Syst Evol Microbiol, 35: 482-488.

Kim Y, Flynn TR, Donoff RB, Wong DTW & Todd R (2002) The gene: the polymerase reaction and its clinical application. J Oral Maxillofac Surg, 60: 808-815.

King JW, Nguyen VQ & Conrad SA (1988) Results of a prospective statewide reporting system for infective endocarditis. Am J Med Sci, 295: 517-527.

Kitada K, Inoue M & Kitano M (1997) Experimental endocarditis induction and platelet aggregation by *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. FEMS Immunol Med Microbiol, 19: 25-32.

Kloos WE & Schleifer KH (1975) Simplified scheme for routine identification of human *Staphylococcus* species. J Clin Microbiol, 1: 82-87.

Kloos WE & Bannerman TL (1994) Update on the clinical significance of coagulase-negative staphylococci. Clin Microbiol Rev, 7: 117-140.

Knox KW & Hunter N (1991) The role of oral bacteria in the pathogenesis of infective endocarditis. Aust Dent J, 36: 286-292.

Kolenbrander PE (2000) Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol, 54: 413-437.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC & Winn WC Jr (1997) Color atlas and textbook of diagnostic microbiology. 5th ed, Lippincott Williams & Wilkins, Philadelphia, PA.

Könönen E, Jousimies-Somer H, Bryk A, Kilp T & Kilian M (2002) Establishment of streptococci in the upper respiratory tract: longitudinal changes in the mouth and nasopharynx up to 2 years of age. J Med Microbiol, 51: 723-730.

Kralovic SM, Melin-Aldana H, Smith KK & Linnemann CC Jr (1995) *Staphylococcus lugdunensis* endocarditis after tooth extraction. Clin Infect Dis, 20: 715-716.

Kremery V & Trupl J (1995) Bacteremia due to penicillin-resistant *Streptococcus viridans* in cancer patients, before and after the prophylaxis with penicillin. Lancet, 346: 1362-1363.

Kroes I, Lepp PW & Relman DA (1999) Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci USA, 96: 14547-14552.

Kuriyama T, Karasawa T, Nakagawa K, Saiki Y, Yamamoto E & Nakamura S (2000) Bacteriologic features and antimicrobial susceptibility in isolates from orofacial odontogenic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 90: 600-608.

Kuriyama T, Karasawa T, Nakagawa K, Yamamato E & Nakamura S (2002) Bacteriology and antimicrobial susceptibility of Gram-positive cocci isolated from pus specimens of odontogenic infections. Oral Microbiol Immunol, 17: 132-135.

Lallemand S, Thouverez M, Boisson K, Talon D & Bertrand X (2002) Bacteraemia caused by coagulase-negative staphylococci exhibiting decreased susceptibility to teicoplanin. J Hosp Infect, 51: 207-214.

Lambe DW Jr, Ferguson KP, Keplinger JL, Gemmel CG & Kalbfleisch JH (1990) Pathogenicity of *Staphylococcus lugdunensis*, *Staphylococcus schleiferi* and three other coagulase-negative staphylococci in a mouse model and possible virulence factors. Can J Microbiol, 36: 455-463.

Landis JR & Koch GG (1977) An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics, 33: 363-374.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML & Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA, 82: 6955-6959.

Larsen T, Fiehn NE, Gutschik E & Bangsborg JM (1999) Current status of taxonomic groups of oral streptococci in endocarditis. Can virulence factors discriminate between endocarditis and non-endocarditis strains? Clin Microbiol Infect, 5: 73-77.

Latorre M, Rojo PM, Franco R & Cisterna R (1993) Endocarditis due to *Staphylococcus capitis* subspecies *urealyticus*. Clin Infect Dis, 16: 343-344.

Lemozy J, Maestre P, Huguet F, Chomarat M, Dabernat H & Lareng MB (1990) Source of infection in *Stomatococcus mucilaginosus* septicaemia. Lancet, 335: 416.

Leviner E, Tzukert AA, Benoliel R, Baram O & Sela MN (1987) Development of resistant oral viridans streptococci after administration of prophylactic antibiotics: time management in the dental treatment of patients susceptible to infective endocarditis. Oral Surg Oral Med Oral Pathol, 64: 417-420.

Levitz RE (1999) Prosthetic-valve endocarditis caused by penicillin-resistant *Streptococcus mitis*. N Eng J Med, 340: 1843-1844.

Levy CS, Kogulan P, Gill VJ, Croxton MB, Kane JG & Lucey DR (2001) Endocarditis caused by penicillin-resistant viridans streptococci: 2 cases and controversies in therapy. Clin Infect Dis, 33: 577-579.

Lewis JF & Abramson JH (1980) Endocarditis due to *Propionibacterium acnes*. Am J Clin Pathol, 74: 690-692.

Ley BE, Linton CJ, Bennet DMC, Jalal H, Foot ABM & Millar MR (1998) Detection of bacteraemia in patients with fever and neutropenia using 16S rRNA gene amplification by polymerase chain reaction. Eur J Microbiol Infect Dis, 17: 247-253.

Li W & Somerville J (1998) Infective endocarditis in the grown-up congenital heart (GUCH) population. Eur Heart J, 19: 166-173.

Li X, Kolltveit KM, Tronstad L & Olsen I (2000) Systemic diseases caused by oral infection. Clin Microbiol Rev, 13: 547-558.

Liljemark WF, Bloomquist CG, Bandt CL, Pihlstrom BL, Hinrichs JE & Wolff JE (1993) Comparison of the distribution of *Actinomyces* in dental plaque on inserted enamel and natural tooth surfaces in periodontal health and disease. Oral Microbiol Immunol, 8: 5-15.

Lina B, Celard M, Vandenesch F, Ribier A, Delahaye JP & Etienne J (1992) Infective endocarditis due to *Staphylococcus capitis*. Clin Infect Dis, 15: 173-174.

Lineberger LT & De Marco TJ (1973) Evaluation of transient bacteraemia following routine periodontal procedures. J Periodontol, 44: 757-763.

Listgarten MA (1994) The structure of dental plaque. Periodontol 2000, 5: 52-65.

Littler WA (2001) Clindamycin suspension and endocarditis prophylaxis. Br Dent J, 190: 407.

Llopis F & Carratala J (2000) Vertebral osteomyelitis complicating *Rothia dentocariosa* endocarditis. Eur J Clin Microbiol Infect Dis, 19: 562-563.

Lockhart PB (1996) An analysis of bacteremias during dental extractions. Arch Intern Med, 156: 513-520.

Lockhart PB, Brennan MT, Kent ML, Norton HJ & Weinrib DA (2004) Impact of amoxicillin on the incidence, nature, and duration of bacteremia in children after intubation and dental procedures. Circulation, 109: 2878-2884.

Loe H & Silness J (1963) Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand, 21: 532-551.

Loesche WJ (1986) Role of *Streptococcus mutans* in human dental decay. Microbiol Rev, 50: 353-380.

Loesche WJ (1997) Association of the oral flora with important medical diseases. Curr Opin Periodontol, 4: 21-28.

Lofthus JE, Waki MY, Jolkovsky DL, Otomo-Corgel J, Newman MG, Flemmig T & Nachnani S (1991) Bacteremia following subgingival irrigation and scaling and root planing. J Periodontol, 62: 602-607.

Lonks JR, Dickinson BP & Runarsdottir V (1999) Endocarditis due to *Streptococcus mitis* with high-level resistance to penicillin and cefotaxime. N Eng J Med, 341: 1239.

Lopez-Velez R, Fortun J, de Pablo C & Martinez Beltran J (1994) Native-valve endocarditis due to *Neisseria sicca*. Clin Infect Dis, 18: 660-661.

Lowbury EJL & Hurst L (1959) The sensitivity of staphylococci and other wound bacteria to erythromycin, oleandomycin, and spiramycin. J Clin Pathol, 12: 163-169.

Lowrance JH, Baddour LM & Simpson WA (1990) The role of fibronectin binding in the rat model of experimental endocarditis caused by *Streptococcus sanguis*. J Clin Invest, 86: 7-13.

Lucas VS, Roberts GJ & Beighton D (1998) Oral health of children undergoing allogeneic bone marrow transplantation. Bone Marrow Transplant., 22: 801-808.

Lucas VS, Gupta R, Ololade O, Gelbier M & Roberts GJ (2000) Dental health indices and caries associated microflora in children with unilateral cleft lip and palate. Cleft Palate Craniofac J, 37: 447-452.

Lucas VS & Roberts GJ (2000) Odontogenic bacteremia following tooth cleaning procedures in children. Pediatr Dent, 22: 96-100.

Lucas VS, Roberts GJ, Greville C and Holzel H (2001) Duration, prevalence and intensity of bacteraemia following dental extraction in children. 6th International Symposium on Endocarditis and Cardiovascular Infections, Barcelona, Abstract 11: 32.

Lucas VS, Lytra V, Hassan T, Tatham H, Wilson M & Roberts GJ (2002a) Comparison of lysis filtration and an automated blood culture system (BACTEC) for detection, quantification, and identification of odontogenic bacteremia in children. J Clin Microbiol, 40: 3416-3420.

Lucas VS, Omar J, Vieira A & Roberts GJ (2002b) The relationship between odontogenic bacteraemia and orthodontic treatment procedures. Eur J Orthod, 24: 293-301.

Mainardi JL, Lortholany O, Buu-Hoi A, Desplaces N, Goldstein F, Gutmann L & Acar JF (1993) Native valve endocarditis caused by *Staphylococcus capitis*. Eur J Clin Microbiol Infect Dis, 12: 789-791.

Manford M, Matharu J & Farrington K (1992) Infective endocarditis in a district general hospital. J R Soc Med, 85: 262-266.

Manning JE, Hume EB, Hunter N & Knox KW (1994) An appraisal of the virulence factors associated with streptococcal endocarditis. J Med Microbiol, 40: 110-114.

Marchant S, Brailsford SR, Twomey AC, Roberts GJ & Beighton D (2001) The predominant micoflora of nursing caries lesions. Caries Res, 35: 397-406.

Mardis JS & Many WJ Jr (2001) Endocarditis due to *Actinomyces viscosus*. South Med J, 94: 240-243.

Marron A, Carratalà J, Gonzalez-Barca E, Fernández-Sevilla A, Alcaide F & Gudiol F (2000) Serious complications of bacteremia caused by viridans streptococci in neutropenic patients with cancer. Clin Infect Dis, 31: 1126-1130.

Marron A, Carratalà J, Fernando A, Fernández-Sevilla A & Gudiol F (2001) High rates of resistance to cephalosporins among viridans-group streptococci causing bacteraemia in neutropenic cancer patients. J Antimicrob Chemother, 47: 87-91.

Marsh PD & Martin MV (1999) Oral microbiology. 3rd ed, Chapman & Hall, London.

Marshall SM, Melito PL, Woodward DL, Johnson WM, Rodgers FG & Mulvey MR (1999) Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. J Clin Microbiol, 37: 4158-4160.

Martin JM, Neches WH & Wald ER (1997) Infective endocarditis: 35 years of experience at a children's hospital. Clin Infect Dis, 24: 669-675.

Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M & Bergeron MG (2001) Development of a PCR assay for identification of staphylococci at genus and species levels. J Clin Microbiol, 39: 2541-2547.

Martínez-Martínez L, Ortega MC & Suárez AI (1995) Comparison of E test with broth microdilution and disk diffusion for susceptibility testing of coryneform bacteria. J Clin Microbiol, 33: 1318-1321.

Maskell J, Sefton A & Williams J (1990) Comparative *in-vitro* activity of azithromycin and erythromycin against Gram-positive cocci, *Haemophilus influenza* and anaerobes. J Antimicrob Chemother, 25 (Suppl A): 19-24.

Masoudi FA & Sande MA (1998) Infective endocarditis In: Internal medicine. 5th ed, MD Stein et al., Eds., Mosby, St. Louis, Missouri, pp. 225-235.

Mathew J, Addai T, Anand A, Morrobel A, Maheshwari P & Freels S (1995) Clinical features, site of involvement, bacteriologic findings, and outcome of infective endocarditis in intravenous drug users. Arch Intern Med, 155: 1641-1648.

Mathisen GE, Meyer RD, George WL, Citron DM & Finegold SM (1984) Brain abscess and cerebritis. Rev Infect Dis, 6: 101-106.

Matsson L (1993) Factors influencing the susceptibility to gingivitis during childhood--a review. Int J Paediatr Dent, 3: 119-127.

Maugein J, Pellegrin JL, Broussard G, Foursche J, Leng B & Reiffers J (2004) *In vitro* activity of vancomycin or teicoplanin against coagulase-negative staphylococci isolated from neutropenic patients. Antimicrob Agent Chemother, 34: 901-903.

McCarthy C, Snyder ML & Parker RB (1965) The indigenous oral flora of Man - I. The newborn to the 1 year old infants. Arch Oral Biol, 10: 61-70.

McGowan DA, Nair S, MacFarlane TW & MacKenzie D (1983) Prophylaxis of experimental endocarditis in rabbits using one or two doses of amoxycillin. Br Dent J, 155: 88-90.

McNab R, Holmes AR, Clarke JM, Tannock GW & Jenkinson HF (1996) Cell-surface polypeptide CshA mediates binding of *Streptococcus gordonii* to other oral bacteria and to immobilized fibronectin. Infect Immun, 64: 4204-4210.

McWhinney PH, Kibbler CC, Gillespie SH, Patel S, Morrison D, Hoffbrand AV & Prentice HG (1992) *Stomatococcus mucilaginosus*: an emerging pathogen in neutropenic patients. Clin Infect Dis, 14: 641-646.

Medlin L, Elwood HJ, Stickel S & Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene, 71: 491-499.

Meyer DJ & Gerding DN (1988) Favorable prognosis of patients with prosthetic valve endocarditis caused by Gram-negative bacilli of the HACEK group. Am J Med, 85: 104-107.

Miele PS, Kogulan PK, Levy CS, Goldstein S, Marcus KA, Smith MA, Rosenthal J, Croxton M, Gill VJ & Lucey DR (2001) Seven cases of surgical native valve endocarditis caused by coagulase-negative staphylococci: an underappreciated disease. Am Heart J, 142: 571-576.

Miyake Y, Iwai T, Sugai M, Miura K, Suginaka H & Nagasaka N (1991) Incidence and characterization of *Staphylococcus aureus* from the tongues of children. J Dent Res, 70: 1045-1047.

Mogi AK, Nishi J & Yoshinaga M (1997) Increased prevalence of penicillin-resistant viridans group streptococci in Japanese children with upper respiratory tract infection treated with β-lactam agents and in those with oncohematologic diseases. Pediatr Infect Dis J, 16: 1140-1144.

Moore WE & Moore LV (1994) The bacteria of periodontal diseases. Periodontol 2000, 5: 66-77.

Moreillon P, Overholser CD, Malinverni R, Bille J & Glauser MP (1988) Predictors of endocarditis in isolates from cultures of blood following dental extractions in rats with periodontal disease. J Infect Dis, 157: 990-995.

Mostaghim D, Millard HD & Arbor A (1975) Bacterial endocarditis: a retrospective study. Oral Surg Oral Med Oral Pathol, 40: 219-233.

Murdoch F, Sammons R & Chapple I (2004) Isolation and characterization of subgingival staphylococci from periodontitis patients and controls. Oral Dis, 10: 155-162.

Mustafa D, Lucas VS, Junod P, Evans R, Mason C & Roberts GJ (2001) The dental health and caries-related microflora in children with craniosynostosis. Cleft Palate Craniofac J, 38: 629-635.

Mylonakis E & Calderwood SB (2001) Infective endocarditis in adults. N Eng J Med, 345: 1318-1330.

Nachnani S, Scuteri A, Newman MG, Avanessian AB & Lomelli SL (1992) E-test: a new technique for antimicrobial susceptibility for periodontal microorganisms. J Periodontol, 63: 576-583.

Naiman RA & Barrow JG (1963) Penicillin-resistant bacteria in the mouths and throats of children receiving continuous prophylaxis against rheumatic fever. Ann Intern Med, 58: 768-772.

National Committee for Clinical Laboratory Standards (2001) Methods for antimicrobial susceptibility testing of anaerobic bacteria: approved standard. 5th ed, NCCLS, Villanova, PA.

National Committee for Clinical Laboratory Standards (2003) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 6th ed, NCCLS, Villanova, PA.

Neeleman C, Klaassen CH, de Valk HA, de Ruiter MT & Mouton JW (2004) Amplified fragment length polymorphism fingerprinting is an effective technique to distinguish *Streptococcus pneumoniae* from other streptococci and an efficient alternative to pulsed-field gel electrophoresis for molecular typing of pneumococci. J Clin Microbiol, 42: 369-371.

Nguyen QV, Kavey RE, Colella C & Weiner LB (2000) Infectious endocarditis caused by *Rothia dentocariosa.* Infect Med, 17: 428-441.

Noubani HM & Kulaylat NA (1989) Infective endocarditis in children. A seven-year experience. J Med Liban, 38: 39-42.

Oakley CM (1987) Controversies in the prophylaxis of infective endocarditis: a cardiological view. J Antimicrob Chemother, 20: 99-104.

Okabe K, Nakagawa K & Yamamoto E (1995) Factors affecting the occurence of bacteraemia associated with tooth extraction. Int J Oral Maxillofac Surg, 24: 239-242.

Okada Y, Kitada K, Takagaki M, Ito HO & Inoue M (2000) Endocardiac infectivity and binding to extracellular matrix proteins of oral *Abiotrophia* species. FEMS Immunol Med Microbiol, 27: 257-261.

Okell CC & Elliott SD (1935) Bacteraemia and oral sepsis with special reference to the aetiology of subacute endocarditis. Lancet, 226: 869-872.

Olsen CH (2003) Review of the use of statistics in infection and immunity. Infect Immun, 71: 6689-6692.

Olsen GJ, Overbeek N, Larsen TL, Marsh MJ, McCaughey MJ, Maciukenas MA, Kuan WM, Macke TJ, Xing Y & Woese CR (1992) The ribosomal database project. Nucleic Acids Res, 20 (Suppl): 2199-2200.

Olsen GJ & Woese CR (1993) Ribosomal RNA - a key to phylogeny. FASEB J, 7: 113-123.

Otten JE, Pelz K & Christmann G (1987) Anaerobic bacteremia following tooth extraction and removal of osteosynthesis plates. J Oral Maxillofac Surg, 45: 477-480.

Overholser CD, Moreillon P & Glauser MP (1988) Experimental endocarditis following dental extractions in rats with periodontitis. J Oral Maxillofac Surg, 46: 857-861.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci, 12: 357-358.

Paley D, Moseley CF, Armstrong P & Prober CG (1986) Primary osteomyelitis caused by coagulase-negative staphylococci. J Pediatr Orthop, 6: 622-626.

Pallasch TJ & Slots J (1996) Antibiotic prophylaxis and the medically compromised patient. Periodontol 2000, 10: 107-138.

Pan YP & Caufield PW (2001) Phenotypic and genotypic diversity of *Streptococcus sanguis* in infants. Oral Microbiol Immunol, 16: 235-242.

Partridge SM (2000) Prosthetic valve endocarditis due to *Streptococcus vestibularis*. J Infect, 41: 284-285.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A & Dewhirst FE (2001) Bacterial diversity in human subgingival plaque. J Bacteriol, 183: 3770-3783.

Patel R, Piper M, Rouse S, Steckelberg JM, Uhl JR, Kohner P, Hopkins MK, Cockerill FR & Kline BC (1998) Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. J Clin Microbiol, 36: 3399-3407.

Pearce C, Bowden GH, Evans M, Fitzsimmons SP, Johnson J, Sheridan MJ, Wientzen R & Cole MF (1995) Identification of pioneer viridans streptococci in the oral cavity of human neonates. J Med Microbiol, 42: 67-72.

Percival RS, Challacombe SJ & Marsh PD (1991) Age-related microbiological changes in the salivary and plaque microflora of healthy adults. J Med Microbiol, 35: 5-11.

Perdikaris GS, Pefanis A, Giamarellou H, Nikolopoulos A, Margaris EP, Donta I, Tsitsika A & Karayiannakos P (1997) Successful single-dose teicoplanin prophylaxis against experimental streptococcal, enterococcal, and staphylococcal aortic valve endocarditis. Antimicrob Agent Chemother, 41: 1916-1921.

Perl TM, Rhomberg PR, Bale MJ, Fuchs PC, Jones RN, Koontz FP & Pfaller MA (1994) Comparison of identification systems for *Staphylococcus epidermidis* and other coagulase-negative *Staphylococcus* species. Diagn Microbiol Infect Dis, 18: 151-155.

Persing DH (1991) Polymerase chain reaction: trenches to benches. J Clin Microbiol, 29: 1281-1285.

Pesci EC, Cottle DL & Pickett CL (1994) Genetic, enzymatic and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect Immun, 62: 2687-2694.

Peters G, Locci R & Pulverer G (1982) Adherence and growth of coagulase-negative staphylococci on surfaces of intravenous catheters. J Infect Dis, 146: 479-482.

Peterson LJ & Peacock R (1976) The incidence of bacteremia in pediatric patients following tooth extraction. Circulation, 53: 676-679.

Peterson MC (2002) Spontaneous bacterial peritonitis from *Streptococcus salivarius* in a compromised host. J Infect, 45: 197-198.

Pfaller MA & Herwaldt LA (1988) Laboratory, clinical and epidemiological aspects of coagulase-negative staphylococci. Clin Microbiol Rev, 1: 281-299.

Pinsky RL, Piscitelli V & Patterson JE (1989) Endocarditis caused by relatively penicillin-resistant *Stomatococcus mucilaginosus*. J Clin Microbiol, 27: 215-216.

Poyart C, Berche P & Trieu-Cuot P (1995) Characterization of superoxide dismutase genes from Gram-positive bacteria by polymerase chain reaction using degenerate primers. FEMS Microbiol Lett, 131: 41-45.

Poyart C, Quesne G, Coulon S, Berche P & Trieu-Cuot P (1998) Identification of streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. J Clin Microbiol, 36: 41-47.

Poyart C, Quesnes G & Trieu-Cuot P (2000) Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J Clin Microbiol, 38: 415-418.

Poyart C, Quesne G, Boumaila C & Trieu-Cuot P (2001) Rapid and accurate species-level identification of coagulase-negative staphylococci using the *sodA* gene as a target. J Clin Microbiol, 39: 4296-4301.

Poyart C, Quesne G & Trieu-Cuot P (2002) Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (*sodA*) sequences: reclassification of *'Streptococcus infantarius subsp. coli'* as *Streptococcus lutetiensis sp. nov.* and of *Streptococcus bovis* biotype 11.2 as *Streptococcus pasteurianus sp. nov.* Int J Syst Evol Microbiol, 52: 1247-1255.

Prabhakar S, Mishra A, Singhal A, Katoch VM, Tyagi JS & Prasad HK (2004) Use of the *hupB* gene encoding a histone-like protien of *Mycobacterium tuberculosis* as a target for detection and differentiation of *M. tuberculosis* and *M. bovis*. J Clin Microbiol, 42: 2724-2732.

Prag J, Kjoller E & Espersen F (1985) *Stomatococcus mucilaginosus* endocarditis. Eur J Clin Microbiol, 4: 422-424.

Pujades RE, Escriva E, Jane J, Argimon J, Fernadez F, Fava P, Galera M & Garau J (1990) Tolerance and efficacy of parenterally administered penicillin-streptomycin and orally administered amoxicillin or penicillin V for prophylaxis of experimentally induced streptococcal endocarditis. Antimicrob Agent Chemother, 34: 321-325.

Rachman C, Kabadjova P, Valcheva R & Prevost H (2004) Identification of *Carnobacterium* species by restriction fragment length polymorphism of the 23S rRNA gene intergenic spacer region and species-specific PCR. Appl Environ Microbiol, 70: 4468-4477.

Raff GW & Gray BM (2004) Aortitis in a child with *Abiotrophia defectiva* endocarditis. Pediatr Infect Dis J, 23: 574-576.

Rajasuo A, Perkki K, Nyfors S, Jousimies-Somer H & Meurman JH (2004) Bacteremia following surgical dental extraction with an emphasis on anaerobic strains. J Dent Res, 83: 170-174.

Rams TE, Feik D & Slots J (1990) Staphylococci in human periodontal diseases. Oral Microbiol Immunol, 5: 29-32.

Ready D, Bedi R, Spratt DA, Mullany P & Wilson M (2003) Prevalence, proportions, and identities of antibiotic-resistant bacteria in the oral microflora of healthy children. Microb Drug Resist, 9: 367-372.

Reimer LG, Wilson ML & Weinstein MP (1997) Update on detection of bacteremia and fungemia. Clin Microbiol Rev, 10: 444-465.

Relman DA, Ruoff K & Ferraro MJ (1987) *Stomatococcus mucilaginosus* endocarditis in an intravenous drug abuser. J Infect Dis, 155: 1080-1082.

Renneberg J, Rieneck K & Gutschik E (1995) Evaluation of Staph ID 32 system and Staph-Zym system for identification of coagulase-negative staphylococci. J Clin Microbiol, 33: 1150-1153.

Renneberg J, Niemann LL & Gutschik E (1997) Antimicrobial susceptibility of 278 streptococcal blood isolates to seven antimicrobial agents. J Antimicrob Chemother, 39: 135-140.

Richard P, Del Valle GA, Moreau P, Milpied N, Felice MP, Daeschler T, Harousseau JL & Richet H (1995) Viridans streptococcal bacteraemia in patients with neutropenia. Lancet, 345: 1607-1609.

Richardson JF, Marples RR & de Saxe MJ (1984) Characters of coagulase-negative staphylococci and micrococci from cases of endocarditis. J Hosp Infect, 5: 164-171.

Ritz HL (1967) Microbial population shifts in developing human dental plaque. Arch Oral Biol, 12: 1561-1568.

Roberts GJ, Radford P & Holt R (1987) Prophylaxis of dental bacteraemia with oral amoxycillin in children. Br Dent J, 162: 179-182.

Roberts GJ, Gardner P & Simmons NA (1992) Optimum sampling time for detection of odontogenic bacteraemia in children. Int J Cardiol, 35: 311-315.

Roberts GJ (1997) Odontogenic bacteraemia in children. MPhil, University of London.

Roberts GJ, Holzel HS, Sury MR, Simmons NA, Gardner P & Longhurst P (1997) Dental bacteremia in children. Pediatr Cardiol, 18: 24-27.

Roberts GJ, Simmons NB, Longhurst P & Hewitt PB (1998a) Bacteraemia following local anaesthetic injections in children. Br Dent J, 185: 295-298.

Roberts GJ, Watts R, Longhurst P & Gardner P (1998b) Bacteraemia of dental origin and antimicrobial sensitivity following oral surgical procedures in children. Pediatr Dent, 20: 28-36.

Roberts GJ (1999) Dentists are innocent! "Everyday" bacteremia is the real culprit: a review and assessment of the evidence that dental surgical procedures are a principal cause of bacterial endocarditis in children. Pediatr Cardiol, 20: 317-325.

Roberts GJ, Gardner P, Longhurst P, Black AE & Lucas VS (2000) Intensity of bacteraemia associated with conservative dental procedures in children. Br Dent J, 188: 95-98.

Roberts GJ, Ramsdale D & Lucas VS (2004) Dental aspects of endocarditis prophylaxis: new recommendations from a working group of the British Cardiac Society Clinical Practice Committee and Royal College of Physicians Clinical Effectiveness Unit, British Cardiac Society Recommendations.www.rcseng.ac.uk/fds.

Roberts RB, Krieger AG, Schiller NL & Gross KC (1979) Viridans streptococcal endocarditis: the role of various species including pyridoxal-dependent streptococci. Rev Infect Dis, 1: 955-966.

Robinson L, Kraus FW, Lazansky JP, Wheeler RE & Johnson V (1950) Bacteremias of dental origin. II. A study of factors influencing occurrence and detection. Oral Surg Oral Med Oral Pathol, 3: 923-926.

Rogosa M, Hampp E, Niven TA, Wagner HN Jr, Driscolle EJ & Baer PN (1960) Blood sampling and cultural studies in the detection of postoperative bacteremia. J Am Dent Assoc, 60: 171-180.

Rosenberg J, Jarvis WR, Abbott SL & Vugia DJ (2004) Emergence of vancomycin-resistant enterococci in San Francisco Bay area hospitals during 1994 to 1998. Infect Control Hosp Epidemiol, 25: 408-412.

Rosser SJ, Alfa MJ, Hoban S, Kennedy J & Harding GK (1999) E test versus agar dilution for antimicrobial susceptibility testing of viridans group streptococci. J Clin Microbiol, 37: 26-30.

Rothman RE, Majmudar MD, Kelen GD, Madico G, Gaydos CA, Walker T & Quinn TC (2002) Detection of bacteraemia in emergency department patients at risk of infective endocarditis using universal 16S rRNA primers in a decontaminated polymerase chain reaction assay. J Infect Dis, 186: 1677-1681.

Ruben SJ (1993) *Rothia dentocariosa* endocarditis. West J Med, 159: 690-691.

Rubin SJ, Lyons RW & Murcia AJ (1978) Endocarditis associated with cardiac catheterization due to a Gram-positive coccus designated *Micrococcus mucilaginosus incertae sedis*. J Clin Microbiol, 7: 546-549.

Rubinstein E & Carbon C (1998) Staphylococcal endocarditis: recommendations for therapy. Clin Microbiol Infect, 4 (Suppl 3): S27-S33.

Rudney JD & Larson CJ (1994) Use of restriction fragment length polymorphism analysis of rRNA genes to assign species to unknown clinical isolates of oral viridans streptococci. J Clin Microbiol, 32: 437-443.

Ruoff KL (1991) Nutritionally variant streptococci. Clin Microbiol Rev, 4: 184-190.

Ruoff KL (2002) Miscellaneous catalase-negative, Gram-positive cocci. Emerging pathogens. J Clin Microbiol, 40: 1129-1133.

Rupp ME & Archer GL (1994) Coagulase-negative staphylococci: pathogens associated with medical progress. Clin Infect Dis, 19: 231-245.

Sabella C, Murphy D & Drummond WJ (2001) Endocarditis due to *Streptococcus mitis* with high-level resistance to penicillin and ceftriaxone. JAMA, 285: 2195.

Sadiq M, Nazir M & Sheikh SA (2001) Infective endocarditis in children--incidence, pattern, diagnosis and management in a developing country. Int J Cardiol, 78: 175-182.

Saiman L, Prince A & Gersony WM (1993) Pediatric infective endocarditis in the modern era. J Pediatr, 122: 847-853.

Salamon SA & Prag J (2002) Three cases of *Rothia dentocariosa* bacteraemia: frequency in Denmark and a review. Scand J Infect Dis, 34: 153-157.

Salventi JF, Davies TA, Randall E, Whitaker S & Waters JR (1979) Effect of blood dilution on recovery of organisms from clinical blood cultures in medium containing sodium polyanethol sulfonate. J Clin Microbiol, 9: 248-252.

Sandoe JAT, Kerr KG, Reynolds GW & Jain S (1999) *Staphylococcus capitis* endocarditis: two cases and review of the literature. Heart, 82: e1-e3.

Santosham M & Moxon ER (1977) Detection and quantitation of bacteremia in childhood. J Pediatr, 91: 719-721.

Sanzén L & Walder M (1988) Antibiotic resistance of coagulase-negative staphylococci in an orthopaedic department. J Hosp Infect, 12: 103-108.

Sarkonen N, Kononen E, Summanen P, Kanervo P, Takala A & Jouisimies-Somer H (2000) Oral colonization of *Actinomyces* species in infants by two years of age. J Dent Res, 79: 864-867.

Sarkonen N, Könönen M, Summanen P, Könönen M & Jousimies-Somer H (2001) Phenotypic identification of *Actinomyces* and related species isolated from human sources. J Clin Microbiol, 39: 3955-3961.

Sato T, Sato M, Matsuyama J, Kalfas S, Sundqvist G & Hoshino E (1998) Restriction fragment-length polymorphism analysis of 16S rDNA from oral asaccharolytic *Eubacterium* species amplified by polymerase chain reaction. Oral Microbiol Immunol, 13: 23-29.

Sato T & Kuramitsu HK (1999) Restriction fragment-length polymorphism analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction for rapid identification of cultivable oral treponemes. Oral Microbiol Immunol, 14: 117-121.

Savelkoul PHM, Aarts HJM, Hass J, Dijkshoorn L, Duim B, Otsen M, Rademaker JLW, Schouls L & Lenstra JA (1999) Amplified-fragment length polymorphism analysis: the state of an art. J Clin Microbiol, 37: 3083-3091.

Schaberg DR, Culver DH & Gaines RP (1991) Major trends in the microbial etiology of nosocomial infection. Am J Med, 91 (Suppl 3B): S72-S75.

Schafer FJ, Wing EJ & Norden CW (1979) Infectious endocarditis caused by *Rothia dentocariosa*. Ann Intern Med, 91: 747-748.

Schlegel L, Grimont F, Collins MD, Regnault B, Grimont PA & Bouvet A (2000) *Streptococcus infantarius* sp. nov., *Streptococcus infantarius* subsp. *infantarius* subsp. nov. and *Streptococcus infantarius* subsp. *coli* subsp. nov., isolated from humans and food. Int J Syst Evol Microbiol, 50: 1425-1434.

Schleifer KH & Kilpper-Balz R (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Bacteriol, 34: 31-34.

Schleifer KH & Kilpper-Balz R (1987) Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci, and lactococci: a review. Syst Appl Microbiol, 10: 1-19.

Schulz R, Werner GS, Fuchs JB, Andreas S, Prange H, Ruschewski W & Kreuzer H (1996) Clinical outcome and echocardiographic findings of native and prosthetic valve endocarditis in the 1990's. Eur Heart J, 17: 281-288.

Sconyers JR, Crawford JJ & Moriarty JD (1973) Relationship of bacteraemia to toothbrushing in patients with periodontitis. J Am Dent Assoc, 87: 616-622.

Seenivasan MH & Yu VL (2003) *Staphylococcus lugdunensis* endocarditis - the hidden peril of coagulase-negative staphylococcus in blood cultures. Eur J Clin Microbiol Infect Dis, 22: 489-491.

Seifert H, Kaltheuner M & Perdreau-Remington F (1995) *Micrococcus luteus* endocarditis: case report and review of the literature. Zentralbl Bakteriol, 282: 431-435.

Sell S (1996) The reticuloendothelial system - blood and tissue clearance In: Immunology, immunopathology and immunity. 5th ed, S Sell et al., Eds., Appleton & Lange, Stamford, Connecticut, pp. 275-288.

Seymour RA, Lowry R, Whitworth JM & Martin MV (2000) Infective endocarditis, dentistry and antibiotic prophylaxis; time for a rethink? Br Dent J, 189: 610-616.

Shahar E, Wohl-Gottesman B-S & Shenkman L (1990) Contamination of blood cultures during venepuncture: fact or myth? Postgrad Med J, 66: 1053-1058.

Shanson DC (1989a) Infection of the heart. In: Microbiology in clinical practice. 2nd ed, Wright, Ed., London, pp. 413-429.

Shanson DC (1989b) Modern blood culture techniques and other methods for detecting microbes in blood In: Septicaemia and endocarditis : Clinical and microbiological aspects. DC Shanson, Ed., Oxford University Press, Oxford, pp. 77-102.

Sheehy EC, Heaton N, Smith P & Roberts GJ (1999) Dental management of children undergoing liver transplantation. Pediatr Dent, 21: 272-280.

Sherman JM (1937) The streptococci. Bacteriol Rev, 1: 3-97.

Shore KP, Pottumarthy S & Morris AJ (1999) Susceptibility of anaerobic bacteria in Auckland: 1991-1998. N Z Med J, 12: 424-426.

Siddiq S, Missri J & Silverman DI (1996) Endocarditis in an urban hospital in the 1990s. Arch Intern Med, 156: 2454-2458.

Silness J & Loe H (1964) Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand, 22: 121-135.

Silver JG, Martin L & McBride BC (1975) Recovery and clearance rates of oral microorganisms following experimental bacteraemias in dogs. Arch Oral Biol, 20: 675-679.

Silver JG, Martin AW & McBride BC (1977) Experimental transient bacteraemias in human subjects with varying degrees of plaque accumulation and gingival inflammation. J Clin Periodontol, 4: 92-99.

Simmons NA (1993) Recommendations for endocarditis prophylaxis. The Endocarditis Working Party for Antimicrobial Chemotherapy. J Antimicrob Chemother, 31: 437-438.

Skehan JD, Murray M & Mills PG (1988) Infective endocarditis: incidence and mortality in the North East Thames Region. Br Heart J, 59: 62-68.

Slots J, Feik D & Rams TE (1990) Age and sex relationships of superinfecting microorganisms in periodontitis patients. Oral Microbiol Immunol, 5: 305-308.

Smith DJ, Anderson JM, King WF, van Houte J & Taubman MA (1993) Oral streptococcal colonization of infants. Oral Microbiol Immunol, 8: 1-4.

Smith MW & Doolittle R (1992) A comparison of evolutionary rates of the two major kinds of superoxide dismutase. J Mol Evol, 34: 175-184.

Sofianou D, Avgoustinakis E, Dilopoulou A, Pournaras S, Tsirakidis G & Tsakris A (2004) Soft-tissue abscess involving *Actinomyces odontolyticus* and two *Prevotella* species in an intravenous drug abuser. Comp Immunol Microbiol Infect Dis, 27: 75-79.

Sommer P, Gleyzal C, Guerret S, Etienne J & Grimaud JA (1992) Induction of a putative laminin-binding protein of *Streptococcus gordonii* in human infective endocarditis. Infect Immun, 60: 360-365.

Sonbol H, Pelargidou M, Lucas VS, Gelbier MJ, Mason C & Roberts GJ (2001) Dental health indices and caries-related microflora in children with severe haemophilia. Haemophilia, 7: 468-474.

Souhami L, Feld R, Tuffnell PG & Feller T (1979) *Micrococcus luteus* pneumonia: a case report and review of the literature. Med Pediatr Oncol, 7: 309-314.

Speck WT, Hurwitz GA & Keller GB (1971) Transient bacteremia in pediatric patients following dental manipulation. Am J Dis Child, 121: 286-288.

Stackebrandt E & Goebel BM (1994) A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol, 44: 846-849.

Steckelberg JM & Wilson WR (1993) Risk factors for infective endocarditis. Infect Dis Clin North Am, 7: 9-19.

Steingrube VA, Wilson RW, Brown BA, Jost KC, Blacklock Z, Gibson JL & Wallace RJ (1997) Rapid identification of clinically significant species and taxa of aerobic actinomycetes, including *Actinomadura*, *Gordona*, *Nocardia*, *Rhodococcus*, *Streptomyces* and *Tsukamurella* isolates by DNA amplificaiton and restriction endonuclease analysis. J Clin Microbiol, 35: 817-822.

Sultana F, Kawamura Y, Gang X, Shu S & Ezaki T (1998) Determination of 23S rRNA sequences from members of the genus *Streptococcus* and characterization of genetically distinct organisms previously identified as members of the *Streptococcus anginosus* group. FEMS Microbiol Lett, 158: 223-230.

Tak T, Reed KD, Haselby RC, McCauley CS, Jr. & Shukla SK (2002) An update on the epidemiology, pathogenesis and management of infective endocarditis with emphasis on *Staphylococcus aureus*. WMJ, 101: 24-33.

Tanner AC, Maiden MF, Paster BJ & Dewhirst FE (1994) The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol 2000, 5: 26-51.

Tanner AC, Milgram PM, Kent R Jr, Mokeem SA, Page RC, Riedy CA, Weinstein P & Bruss J (2002) The microbiota of young children from tooth and tongue samples. J Dent Res, 81: 53-57.

Tappuni AR & Challacombe SJ (1993) Distribution and isolation frequency of eight streptococcal species in saliva from predentate and dentate children and adults. J Dent Res, 72: 31-36.

Tardif G, Sulavik MC, Jones GW & Clewell DB (1989) Spontaneous switching of the sucrose-promoted colony phenotype in *Streptococcus sanguis*. Infect Immun, 57: 3945-3948.

Tariq M, Alam M, Munir G, Khan MA & Smego RA, Jr. (2004) Infective endocarditis: a five-year experience at a tertiary care hospital in Pakistan. Int J Infect Dis, 8: 163-170.

Taubert KA & Dajani AS (2001) Optimisation of the prevention and treatment of bacterial endocarditis. Drugs Aging, 18: 415-424.

Teng LJ, Hsueh PR, Chen YC, Ho SW & Luh KT (1998) Antimicrobial susceptibility of viridans group streptococci in Taiwan with an emphasis on the high rate of resistance to penicillin and macrolides in *Streptococcus oralis*. J Antimicrob Chemother, 41: 621-627.

Tenover FC, Swenson JM, O'Hara CM & Stocker SA (1995) Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci. J Clin Microbiol, 33: 1524-1527.

Tenover FC, Baker CN & Swenson JM (1996) Evaluation of commercial methods for determining antimicrobial susceptibility of *Streptococcus pneumoniae*. J Clin Microbiol, 34: 10-14.

Terada Y, Mitsui T & Enomoto Y (1996) Prosthetic valve endocarditis caused by *Staphylococcus capitis*. Ann Thorac Surg, 62: 317-324.

Theilade E, Fejerskov O, Karring T & Theilade J (1982) Predominant cultivable microflora of human dental fissure plaque. Infect Immun, 36: 977-982.

Thompson J, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The ClustalX windows interface: flexible strategies multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res, 25: 4876-4882.

Thornhill-Joynes M & Li MW (1985) *Neisseria sicca* endocarditis in intravenous drug abusers. West Med J, 142: 255-256.

Tiley SM, Kociuba KR, Heron LG & Munro R (1993) Infective endocarditis due to nontoxigenic *Corynebacterium diphtheriae*: report of seven cases and review. Clin Infect Dis, 16: 271-275.

Tilton RC (1982) The laboratory approach to the detection of bacteremia. Annu Rev Microbiol, 36: 467-493.

Titsas A & Ferguson MM (2001) Concepts for the prophylaxis of infective endocarditis in dentistry. Aust Dent J, 46: 220-225.

Tong H, Gao X & Dong X (2003) *Streptococcus oligofermentans* sp. nov., a novel oral isolate from caries-free humans. Int J Syst Evol Microbiol, 53: 1101-1104.

Traub WH & Kleber I (1977) Inactivation of classical and alternative pathway-activated bactericidal activity of human serum by sodium polyanetholsulfonate. J Clin Microbiol, 5: 278-284.

Tronel H, Chaudemanche H, Pechier N, Doutrelant L & Hoen B (2001) Endocarditis due to *Neisseria mucosa* after tongue piercing. Clin Microbiol Infect, 7: 275-276.

Tsitsika A, Pefanis A, Perdikaris GS, Donta I, Karayiannakos P & Giamarellou H (2000) Single-oral-dose azithromycin prophylaxis against experimental streptococcal or staphylococcal aortic valve endocarditis. Antimicrob Agent Chemother, 44: 1754-1756.

Tuazon CU, Gill V & Gill F (1986) Streptococcal endocarditis: single vs. combination antibiotic therapy and role of various species. Rev Infect Dis, 8: 54-60.

Udo EE, Jacob LE & Chugh TD (1995) Antimicrobial resistance of coagulase-negative staphylococci from a Kuwait hospital. Microb Drug Resist, 1: 315-320.

Uh Y, Jeong SS, Jang IH, Yoon KJ & Hong SK (2002) Penicillin-resistant *Aerococcus viridans* bacteraemia associated with granulocytopenia. J Korean Med Sci, 17: 113-115.

Van der Meer JTM, van Vianen W, Hu E, van Leeuwen WB, Valkenburg HA, Thompson J & Michel MF (1991) Distribution, antibiotic susceptibility and tolerance of bacterial isolates in culture-positive cases of endocarditis in The Netherlands. Eur J Clin Microbiol Infect Dis, 10: 728-734.

Van der Meer JTM, Thompson J, Valkenburg HA & Michel MF (1992) Epidemiology of bacterial endocarditis in The Netherlands. II. Antecedent procedures and use of prophylaxis. Arch Intern Med, 152: 1869-1873.

Van der Meer JTM (2002) Prophylaxis of endocarditis. Neth J Med, 60: 423-427.

Van Hare GF, Ben Shachar G, Liebman J, Boxerbaum B & Riemenschneider TA (1984) Infective endocarditis in infants and children during the past 10 years: a decade of change. Am Heart J, 107: 1235-1240.

Vandenesch F, Lebeau C & Bes M (1994) Clotting activity in *Staphylococcus schleiferi* subspecies from human patients. J Clin Microbiol, 32: 388-392.

Vernaleo JR, Cleri DJ, Joachim GR, Mastellone AJ, Wallman AA & Perlman J (1992) *Neisseria sicca* endocarditis with embolic phenomenon. Diagn Microbiol Infect Dis, 15: 165-167.

Von Graevenitz A (2004) *Rothia dentocariosa*: taxonomy and differential diagnosis. Clin Microbiol Infect, 10: 399-402.

Voorn GP, Thompson J, Goessens WHF, Schmall-Bauer WC, Broeders PHM & Michel MF (1992) Role of tolerance in cloxacillin prophylaxis of experimental *Staphylococcus aureus* endocarditis. J Infect Dis, 166: 169-173.

Wahlmann U, Al Nawas B, Jutte M & Wagner W (1999) Clinical and microbiological efficacy of single dose cefuroxime prophylaxis for dental surgical procedures. Int J Antimicrob Agents, 12: 253-256.

Washington JA & Ilstrup JA (1986) Blood culture: issues and controversies. Rev Infect Dis, 8: 792-802.

Watanakunakorn C (1994) *Staphylococcus aureus* endocarditis at a community teaching hospital, 1980 to 1991. An analysis of 106 cases. Arch Intern Med, 154: 2330-2335.

Weinstein MP (1996) Current blood culture methods and systems: clinical concepts, technology, and interpretation of results. Clin Infect Dis, 23: 40-46.

Weinstein MP, Mirrett S, Van Pelt L, McKinnon M, Zimmer BL, Kloos WE & Reller LB (1998) Clinical importance of identifying coagulase-negative staphylococci isolated from blood cultures: evaluation of MicroScan Rapid and Dried Overnight Gram-positive panels versus a conventional reference method. J Clin Microbiol, 36: 2089-2092.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol, 173: 697-703.

Welch DF, Scribner RK & Hensel D (1985) Evaluation of a lysis direct plating method for pediatric blood cultures. J Clin Microbiol, 21: 955-958.

Weller TM, Crook DW, Crow MR, Ibrahim W, Pennington TH & Selkon JB (1997) Methicillin susceptibility testing of staphylococci by E test and comparison with agar dilution and mecA detection. J Antimicrob Chemother, 39: 251-253.

Welsby PD (1977) Infective endocarditis - a retrospective study. Practitioner, 218: 382-387.

West PWJ, Al-Sawan R, Foster HA, Electricwala Q, Alex A & Panigrahi D (1998) Speciation of presumptive viridans streptococci from early onset neonatal sepsis. J Med Microbiol, 47: 923-928.

Whiley RA & Hardie JM (1988) *Streptococcus vestibularis* sp. nov. from the human oral cavity. Int J Syst Bacteriol, 38: 335-339.

Whiley RA, Russell RRB, Hardie JM & Beighton D (1988) *Streptococcus downei* sp. nov. for strains previously described as *Streptococcus mutans* serotype h. Int J Syst Evol Microbiol, 38: 25-29.

Whiley RA & Hardie JM (1989) DNA-DNA hybridization studies and phenotypic characteristics of strains within the 'Streptococcus milleri group'. J Gen Microbiol, 135: 2623-2633.

Whiley RA, Fraser H, Hardie JM & Beighton D (1990a) Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the 'Streptococcus milleri group'. J Clin Microbiol, 28: 1497-1501.

Whiley RA, Fraser HY, Douglas CW, Hardie JM, Williams AM & Collins MD (1990b) *Streptococcus parasanguis* sp. nov., an atypical viridans streptococcus from human clinical specimens. FEMS Microbiol Lett, 56: 115-121.

Whiley RA & Beighton D (1998) Current classification of the oral streptococci. Oral Microbiol Immunol, 13: 195-216.

White JC & Niven JCF (1946) Streptococcus SBE: a streptococcus associated with subacute endocarditis. J Bacteriol, 51: 717-722.

Whyman RA & MacFadyen EE (1994) Dens in dente associated with infective endocarditis. Oral Surg Oral Med Oral Pathol, 78: 47-50.

Willcox MD, Loo CY, Harty DWS & Knox KW (1995) Fibronectin binding by *Streptococcus milleri* group strains and partial characterisation of the fibronectin receptor of the fibronectin receptor of *Streptococcus anginosus*. Microb Pathog, 19: 129-137.

Willcox MD, Zhu H & Knox KW (2001) *Streptococcus australis* sp. nov., a novel oral streptococcus. Int J Syst Evol Microbiol, 51: 1277-1281.

Wilson M, McNab R & Henderson B (2002) An introduction to bacterial diseases In: Bacterial disease mechanisms M Wilson et al., Eds., Cambridge University Press, Cambridge, pp. 1-44.

Wisplinghoff H, Reinert RR, Cornely O & Seifert H (1999) Molecular relationships and antimicrobial susceptibilities of viridans group streptococci isolated from blood of neutropenic cancer patients. J Clin Microbiol, 37: 1876-1880.

Woese CR (1987) Bacterial evolution. Microbiol Rev, 51: 221-271.

Wong JD & Janda M (1992) Association of an important *Neisseria* species, *Neisseria elongata* subsp. *nitroreducens*, with bacteremia, endocarditis and osteomyelitis. J Clin Microbiol, 30: 719-720.

Woo PC, Fung AM, Lau SK & Yuen KY (2002) Identification by 16S rRNA gene sequencing of *Lactobacillus salivarius* bacteremic cholecystitis. J Clin Microbiol, 40: 265-267.

Woo PC, Tse H, Chan KM, Lau SK, Fung AM, Yip KT, Tam DM, Ng KH, Que TL & Yuen KY (2004) 'Streptococcus milleri' endocarditis caused by *Streptococcus anginosus*. Diagn Microbiol Infect Dis, 48: 81-88.

Wood CA, Sewell DL & Strausbaugh LJ (1989) Vertebral osteomyelitis and native valve endocarditis caused by *Staphylococcus warneri*. Diagn Microbiol Infect Dis, 12: 261-263.

Wright AJ & Wilson WR (1982) Experimental animal endocarditis. Mayo Clin Proc, 57: 10-14.

Ximénez-Fyvie LA, Haffajee AD & Socransky SS (2000) Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. J Clin Periodontol, 27: 722-732.

Yagupsky P & Nolte FS (1990) Quantitative aspects of septicemia. Clin Microbiol Rev, 3: 269-279.

Young SEJ (1987) Aetiology and epidemiology of infective endocarditis in England and Wales. J Antimicrob Chemother, 20 (Suppl A): 7-15.

Zakrzewska-Czerwinska J, Gaszewska-Mastalarz A, Pulverer G & Mordarski M (1992) Identification of *Staphylococcus epidermidis* using a 16S rRNA-directed oligonucleotide probe. FEMS Microbiol Lett, 79: 51-58.

Zhu H, Willcox MD & Knox KW (2000) A new species of oral *Streptococcus* isolated from Sprague-Dawley rats, *Streptococcus orisratti* sp. nov. Int J Syst Evol Microbiol, 50: 55-61.

Zierdt CH (1982) Blood-lysing solution nontoxic to pathogenic bacteria. J Clin Microbiol, 15: 172-174.

Zierdt CH, Peterson DL, Swan JC & MacLowry JD (1982) Lysis filtration blood culture versus conventional blood culture in a bacteremic rabbit model. J Clin Microbiol, 15: 74-77.

APPENDIX 1
Ethical Approval from the Joint Research and Ethics Committee

EASTMAN DENTAL HOSPITAL

Eastman Dental Institute and Hospital
256 Gray's Inn Road, London WC1X 8LD

Telephone: 0171 915 1000 (Switchboard)
Fax: 0171 915 1012 WWW: www.eastman.ucl.ac.uk

EDI/EDH Joint Research & Ethics Committee
Chairman: Dr R A Jeffreys OBE
Administration: Philip Diamond
2nd Floor, Victoria Wing, Eastman Dental Hospital
Direct Line: 020 7380 9833
or via UCLH R&D Directorate Office
Direct Line: 020 7380 9833
Fax: 020 7380 9937
philip.diamond@uclh.org

02 February 2001

Mrs Hawazen
Department of Paediatric Dentistry
Eastman Dental Hospital

Dear Mrs Hawazen,

JREC Application no. **00/E039** *(Please quote in any correspondence)*
Bacteraemia following conservative dentogingival manipulation procedures in children

Thank you for your recent application to the JREC regarding the above study. Your application has now been approved

Please note that it is important that you notify the Committee of any adverse events or changes (name of investigator etc) relating to this project. You should also notify the Committee on completion of the project, or indeed if the project is abandoned. **Please remember to quote the above number in any correspondence.**

With best wishes,

Yours sincerely

Dr R A Jeffreys OBE
Acting Chairman, JREC

APPENDIX 2
Ethical Approval from the Research and Development Directorate

UCL HOSPITALS

The University College London Hospitals
Research and Development Directorate

15 January 2001

Mrs Sonbol
Department of Paediatric Dentistry
Eastman Dental Hospital

Dear Mrs Sonbol,

Study No: **00/E039** *(Please quote in all correspondence)*
Title: **Bacteraemia following conservative dentogingival manipulation procedures in children**

Thank you for registering the above study with the R&D Directorate. I am pleased to give Trust approval for the study. Please ensure you have addressed any outstanding issues raised by the ethics committee before you start your project.

With best wishes.

Yours sincerely

Professor Allyson Pollock

1st Floor Vezey Strong Wing; 112 Hampstead Rd; London NW1 2LT; Tel: 020 7380 9833..... Fax. 020 7380 9937

APPENDIX 3

Information Sheet
Dental Bacteraemia Study

We are interested in helping children who are born with severe heart defects that affect them for the rest of their lives. To do this we need help from healthy children like yours.

Children born with a hole in the heart or other similar heart defects sometimes have open heart surgery as an infant to repair the defect. This leaves a small scar in the heart. When the children are older this may cause problems following dental treatment. This is because following dental treatment germs (bacteria) may enter the bloodstream from the mouth. These germs sometimes stick to the scar and cause a very serious infection called Bacterial Endocarditis. This infection does not happen to healthy children (like yours) when they have dental treatment.

To study this problem we need the help of you and your child. The Ethical Committee of the Hospital has given us permission to ask parents if they would be willing to let us take 2 small blood samples during your child's care.

This means that the dental treatment would be carried out normally. Once your child has been anaesthetised (been put to sleep) we would put a small sterile tube into a blood vessel in the arm or hand. A small sample of blood would be taken. The dental treatment will then be carried out. A 2nd sample of blood will be taken 30 seconds after either drilling or placing a metal ring around the tooth to pack the filling or placing a sheet of rubber over the tooth to keep it dry or placing a cord around the gum to keep the tooth clear.

This research would help us look after children with birth defects of the heart.

If you want more information please ask to speak to Mrs. Hawazen Sonbol on extension 1108 or Dr. Victoria Lucas on extension 1263 or Professor Graham Roberts on extension 1022.

Frequently asked questions

1. Does it hurt? – No, because blood samples will be taken when the child is fast asleep.
2. Will it cause my child any extra stress? – No, because the sterile tube is put in the arm after the child has gone to sleep.
3. Will there be any complications? – There is a small risk of a bruise at the site of blood sampling.
4. Will it make any difference if I don't agree? – No, your dental treatment will be carried out in the normal way.
5. Will I be required to stay longer than normal? – No.
6. Will it prolong the treatment? – Yes, but only by a few minutes.
7. Will it alter the treatment my child is due to have? – No.

APPENDIX 4

CONSENT FORM FOR PARENTS OR GUARDIANS OF CHILDREN PARTICIPATING IN RESEARCH STUDIES

Study of Bacteria in Blood Following Dental Fillings

DEPARTMENT OF PAEDIATRIC DENTISTRY
EASTMAN DENTAL INSTITUTE AND HOSPITAL

Please read this form carefully and ask if you do not understand or would like more information.

Title of research: Bacteraemia Following Conservative Dento-gingival Manipulative Procedures in Children

Name of investigator: Hawazen Sonbol

I .....(Full name)

Of .....(Address)

Hereby fully and freely consent to the participation of my child

..... in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage and this will in no way affect the care my child receives as a patient.

SIGNED (Parent) _____ Date _____

Dentist _____ Date _____

APPENDIX 5

SOLUTIONS USED FOR LYSIS FILTRATION

SPS solution:

6. Dissolve 0.2 g SPS in 50 ml distilled water
7. Filter sterilize into sterile universals
8. Aliqout 1.23 ml into sterile universals

Lysis Solution:

To make 5 bottles, 190 ml each:

9. Weigh 0.76 g sodium carbonate
10. Dissolve in 950 ml distilled water
11. Adjust to pH 10 using 5 M HCL
12. Dispense 190 ml into a clean 500 ml Duran bottle
13. Add approx. 100 µl (2 drops) of Triton-X-100 to each bottle
14. Autoclave for 15 minutes at 121°C. Solution appears cloudy when just autoclaved
15. Store at room temperature until use
16. Warm solution to 37°C prior to use

APPENDIX 6

Example of Calculation of Total Bacterial Intensity

Assuming

Aerobic count of 300 (200 facultatives + 100 aerobes)

Anaerobic count of 300 (200 facultatives + 100 anaerobes)

Adding both aerobic and anerobic and dividing by 6 ml would give

300 + 300/6 = **100 cfu/ml**

In reality

Facultatives + Aerobes + Anaerobes

(200 +200)/6 + 100/3 + 100/3 = **133.33 cfu/ml**

APPENDIX 7

Example of 16S rRNA Gene Sequence Alignments from BLAST

(*Rothia mucilaginosus*)

Query= (348 letters)

Sequences producing significant alignments	Score (bits)	E Value
gi|1051158|emb|X87758.1|MM16SRRN1 M.mucilaginosus 16S rRNA	628	e-177
gi|54111671|gb|AY753388.1| Uncultured bacterium SK14 16S ri.	599	e-168
gi|34850690|emb|AJ583197.1| uncultured Actinobacterium part...	597	e-168
gi|2764819|emb|X95483.1|SM16SRR S.mucilaginosus 16S rRNA gene	557	e-156

Alignments

>gi|1051158|emb|X87758.1|MM16SRRN1 M.mucilaginosus 16S rRNA gene
Length = 1467

Score = 628 bits (317), Expect = e-177
Identities = 339/344 (98%), Gaps = 2/344 (0%)
Strand = Plus / Plus

```
Query: 5   attgcacaatgggcagcaagcctgatgcagcagacgccgcgtgagggatgacggccttcg 64
           ||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 349 attgcacaatgggc-gcaagcctgatgcagc-gacgccgcgtgagggatgacggccttcg 406


Query: 65  ggttgtaaacctctgttagcagggaagaagagaaattgacggtacctgcagagaaagcgc 124
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 407 ggttgtaaacctctgttagcagggaagaagagagattgacggtacctgcagagaaagcgc 466


Query: 125 cggctaactacgtgccagcagccgcggtaatacgtagggcgcgagcgttgtccggaatta 184
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 467 cggctaactacgtgccagcagccgcggtaatacgtagggcgcgagcgttgtccggaatta 526


Query: 185 ttgggcgtaaagagcttgtaggcggtttgtcgcgtctgctgtgaaaggccggggcttaac 244
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 527 ttgggcgtaaagagcttgtaggcggtttgtcgcgtctgctgtgaaaggccggggcttaac 586


Query: 245 cccgtgtattgcagtgggtacgggcagactagagtgcagtaggggagactggaactcctg 304
           |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct: 587 cccgtgtattgcagtgggtacgggcagactagagtgcagtaggggagactggaattcctg 646


Query: 305 gtgtancggtggaatgcgcagatatcaggaagaacaccgatggc 348
           ||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 647 gtgtagcggtggaatgcgcagatatcaggaagaacaccgatggc 690
```

APPENDIX 8

Example of *sodA* Gene Sequence Alignment from BLAST for Streptococci (*S. salivarius*)

Query= (417 letters)

Sequences producing significant alignments:		Score (bits)	E Value
gi|2765892|emb|Z99199.1|SSZ99199	Streptococcus salivarius p...	795	0.0
gi|2765888|emb|Z99197.1|SSZ99197	Streptococcus salivarius p...	795	0.0
gi|2293086|emb|Z95917.1|SSZ95917	Streptococcus salivarius s...	795	0.0
gi|2765890|emb|Z99198.1|SSZ99198	Streptococcus salivarius p...	668	0.0
gi|2293084|emb|Z95916.1|SSZ95916	Streptococcus salivarius s...	668	0.0
gi|55737978|gb|CP000024.1|	Streptococcus thermophilus CNRZ1...	496	e-137
gi|24182471|gb|AF538722.2|	Streptococcus thermophilus AO54 ...	488	e-135
gi|55736088|gb|CP000023.1|	Streptococcus thermophilus LMG 1...	488	e-135
gi|2293094|emb|Z95921.1|STZ95921	Streptococcus thermophilus...	484	e-134
gi|2293096|emb|Z95922.1|SVZ95922	Streptococcus vestibularis...	474	e-131

Alignments

>gi|2765892|emb|Z99199.1|SSZ99199 Streptococcus salivarius partial sodA gene, strain NEM1257
Length = 435

Score = 795 bits (401), Expect = 0.0
Identities = 413/416 (99%), Gaps = 1/416 (0%)
Strand = Plus / Plus

```
Query: 1   tgactcttc-ccacgataaacaccatgcaacttatgtagcaaatgctaatgctgctcttg 59
           ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 20  tgactcttcaccacgataaacaccatgcaacttatgtagcaaatgctaatgctgctcttg 79

Query: 60  aaaaacacccagaaattggtgaagacattgaagcacttttggctgatgtggaacaaatcc 119
           |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct: 80  aaaaacacccagaaattggtgaagaccttgaagcacttttggctgatgtggaacaaatcc 139

Query: 120 cagctgatattcgtcaagcacttattaataatggnggtggacaccttaaccacgcacttt 179
           |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 140 cagctgatattcgtcaagcacttattaataatggtggtggacaccttaaccacgcacttt 199

Query: 180 tctgggaacttttgtctcctgaaaaacaagaaccaactgctgaagtagcagctgctatta 239
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 200 tctgggaacttttgtctcctgaaaaacaagaaccaactgctgaagtagcagctgctatta 259

Query: 240 acgaagctttcggatcatttgaagccttccaagaagctttcacagcagcagcaacaactc 299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 260 acgaagctttcggatcatttgaagccttccaagaagctttcacagcagcagcaacaactc 319

Query: 300 gttttggttcaggttgggcatggcttgttgttaacgatgaaggtaaacttgaagttgttt 359
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 320 gttttggttcaggttgggcatggcttgttgttaacgatgaaggtaaacttgaagttgttt 379

Query: 360 caactgctaaccaagacactcctatctcagacggtaagaaaccaatcttggcgctt 415
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 380 caactgctaaccaagacactcctatctcagacggtaagaaaccaatcttggcgctt 435
```

APPENDIX 9

Example of *sodA* Gene Sequence Alignments from BLAST for CNS (*Staph epidermidis*)

Query= (390 letters)

Sequences producing significant alignments:		Score (bits)	E Value
gi|27315631|gb|AE016748.1|	Staphylococcus epidermidis ATCC ...	728	0.0
gi|15825380|gb|AF410177.1|	Staphylococcus epidermidis super...	728	0.0
gi|57636010|gb|CP000029.1|	Staphylococcus epidermis RP62A...	728	0.0
gi|18077071|emb|AJ343947.1|SEP343947	Staphylococcus epiderm...	724	0.0
gi|18077069|emb|AJ343946.1|SEP343946	Staphylococcus epiderm...	724	0.0
gi|18076995|emb|AJ343906.1|SEP343906	Staphylococcus epiderm...	724	0.0
gi|18077073|emb|AJ343948.1|SEP343948	Staphylococcus epiderm...	708	0.0
gi|18077059|emb|AJ343941.1|SCA343941	Staphylococcus capitis...	422	e-115
gi|18077057|emb|AJ343940.1|SCA343940	Staphylococcus capitis...	422	e-115

>gi|27315631|gb|AE016748.1| Staphylococcus epidermidis ATCC 12228, section 5 of 9 of the complete genome

Length = 300029

Score = 728 bits (367), Expect = 0.0
Identities = 370/371 (99%)
Strand = Plus / Minus

```
Query: 12    aaattaaattcagcagttgaagggacagatttagaagctaaatcaatcgaagaaattgtt 71
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65813 aaattaaattcagcagttgaagggacagatttagaagctaaatcaatcgaagaaattgtt 65754


Query: 72    gctaatttagatagtgtgccatctaatattcaaacagctgttcgtaataatggcggtggt 131
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65753 gctaatttagatagtgtgccatctaatattcaaacagctgttcgtaataatggcggtggt 65694


Query: 132   caccttaaccattcattgttctgggaactattatcaccaaattctgaagaaaaaggtgaa 191
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65693 caccttaaccattcattgttctgggaactattatcaccaaattctgaagaaaaaggtgaa 65634


Query: 192   gtagtagataaaattaaagaacaatggggttctttagatgaatttaaaaaagaatttgca 251
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65633 gtagtagataaaattaaagaacaatggggttctttagatgaatttaaaaaagaatttgca 65574


Query: 252   gataaagctgcagcacgctttggttcaggaggggcttggttagttgtaaacaatggacaa 311
              |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 65573 gataaagctgcagcacgctttggttcaggatgggcttggttagttgtaaacaatggacaa 65514


Query: 312   ttagaaattgttacaacaccaaatcaagataatccaattactgaaggaaaaacaccaatt 371
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 65513 ttagaaattgttacaacaccaaatcaagataatccaattactgaaggaaaaacaccaatt 65454


Query: 372   ttaggtttaga 382
              ||||||||||
Sbjct: 65453 ttaggtttaga 65443
```

PUBLICATIONS

ABSTRACTS

Prevalence and intensity of bacteraemia following conservative dento-gingival manipulative procedures in children. International Journal of Paediatric Dentistry 2003; 13: Abstract 25; 391.

Bacteraemia following conservative dento-gingival manipulative procedures in children. International Journal of Paediatric Dentistry 2003; Suppl 1: SP-P-3:25

Prevalence and intensity of bacteraemia following conservative dento-gingival manipulative procedures in children. 7th International Symposium on Endocarditis and Cardiovascular Infections, Chamonix, June 2003; Abstract 43